

**PROTEOMIC ANALYSIS OF DOPAMINE OXIDATION INDUCED MODIFICATIONS
TO MITOCHONDRIAL PROTEINS: IMPLICATIONS FOR PARKINSON'S DISEASE**

by

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Parkinson's disease (PD) neurodegeneration is characterized by loss of the dopaminergic cells of the substantia nigra, and has been linked to oxidative stress and mitochondrial dysfunction. The reactive neurotransmitter dopamine (DA) may play a role in neuronal vulnerability. DA oxidation has been shown to elicit dopaminergic toxicity in animal models, covalently modify proteins, and affect mitochondrial function. However, mitochondrial protein targets of DA modification are unknown. In this study, I utilized proteomic techniques to identify and characterize mitochondrial proteins altered following *in vitro* exposure to DA oxidation. Using two-dimensional difference in-gel electrophoresis and mass spectrometry analyses, I identified a subset of mitochondrial proteins that exhibited decreased abundance following exposure of isolated rat brain mitochondria to DA quinone (DAQ). Losses of two of these proteins, mitochondrial creatine kinase (MtCK) and mitofilin were further confirmed by Western blot analyses. Western blot also confirmed significant decreases of these two proteins in differentiated PC12 cells exposed to DA. I next utilized two-dimensional gel electrophoresis with autoradiography to identify proteins covalently modified by DAQ. I identified a subset of proteins covalently modified by ^{14}C -DA from rat brain mitochondria exposed to ^{14}C -DAQ and from differentiated SH-SY5Y cells exposed to ^{14}C -DA. Proteins including

mortalin/GRP75/mtHSP70, subunits of Complex I, MtCK, and mitofilin, amongst other proteins, were found to be covalently modified. We chose to further examine mitofilin, a protein implicated in maintaining mitochondrial structure. To characterize the effect of altered mitofilin levels on cell viability, I utilized overexpression and knockdown techniques to modulate mitofilin expression in dopaminergic cell lines, differentiated PC12 and SH-SY5Y cells, and examined their response to dopaminergic toxins, DA and rotenone. I found that increased mitofilin expression was protective against both DA- and rotenone-induced toxicity in both cell lines, and decreased mitofilin enhanced DA-induced toxicity in differentiated SH-SY5Y cells. Therefore, in this thesis, I identified a subset of mitochondrial and cellular proteins that are potential targets of DA-induced modification, and may have roles in PD pathogenesis. Modulating the expression level of one of these proteins, mitofilin, affected the cellular response to toxins, and may play a role in dopaminergic cell vulnerability.

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PREFACE

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1.0 INTRODUCTION

1.1 PARKINSON'S DISEASE

Parkinson's disease (PD) was first described in 1817 by James Parkinson in "An Essay of the Shaking Palsy" (Parkinson, 1817). In the nearly 200 years that have passed since recognition of this neurological disorder, great strides have been made to characterize and identify disease pathology, clinical symptoms, and therapeutic treatment, but a cure has yet to be identified. Epidemiological studies and laboratory research have long sought to find potential causes of this prevalent disease (Khandhar and Marks, 2007). Though the underlying mechanism of PD pathogenesis remains elusive, several environmental and genetics factors have been linked to PD, suggesting agents and biological pathways that promote PD pathogenesis. Identifying and understanding the etiology of PD progression is key to development of new therapeutics for disease treatment.

1.1.1 Clinical and Pathological Characteristics of PD

Parkinson's disease is not selective for race or region. The overall prevalence of PD has been estimated by various studies, and varies somewhat depending on methodology (de Lau et al., 2004; Khandhar and Marks, 2007). Age appears to be a factor in disease prevalence, with rates of 1-2% in persons over 65 years of age and increasing to 4-5% or higher after age 85 (de

Lau et al., 2004; reviewed in Weintraub et al., 2008). Additionally, studies suggest that prevalence rates are higher for men than women (de Lau et al., 2004; Van Den Eeden et al., 2003).

Clinical symptoms of the disorder typically include a range of movement disorders, postural deficits, and non-motor symptoms. Cardinal motor features include slow movement (bradykinesia), rigidity, and resting tremor. Patients also exhibit postural instability and slow, shuffled gait when walking. Most patients (75-90%) present initially with an asymmetric resting tremor in the upper distal extremity, presenting as a “pill-rolling” motion of the forefinger and thumb (Pallone, 2007; Weintraub et al., 2008). Non-motor symptoms involved in PD include autonomic dysfunction, impacting cardiovascular, gastrointestinal, and bladder function, among others (Pallone, 2007; Weintraub et al., 2008). Neuropsychiatric symptoms, including depression and dementia, impulse control disorders, sleep disorders, and marked olfactory dysfunction are also associated with PD (Barbas, 2006; Pallone, 2007; Weintraub et al., 2008; Weintraub and Stern, 2005). Thus, while motor deficits can be among the more debilitating symptoms of the disease (Pallone, 2007), PD is clearly a disease impacting function and quality of life for the entire body and mind.

Multiple brain regions are involved in PD, as noted by the development of proteinaceous cytoplasmic inclusions called Lewy bodies in select neuronal types and populations. Lewy body deposition in association with PD appears before motor symptoms, and initiates in the dorsal motor nucleus and olfactory bulb regions of the brain (Braak et al., 2003; Braak et al., 2004). Lewy body formation progresses through the locus coeruleus, substantia nigra, and eventually involves the mesocortex and cortex, with long-axon, high-energy expenditure neurons being

particularly vulnerable (Braak et al., 2003; Braak et al., 2004). Thus, PD is a progressive disease involving the entire brain.

The hallmark pathology of PD, and the major contributor to the debilitating motor symptoms of the disease, is the loss of pigmented dopaminergic neurons in the substantia nigra pars compacta (SN) and their projections to the caudate and putamen (reviewed in Samii et al., 2004). This neuron loss ultimately leads to significantly decreased striatal dopamine (DA) levels, with reduction by 60% to 70% from normal levels at the time of diagnosis (Schapira, 2006) corresponding with movement disorders early in the disease. At death, DA loss was found to vary in specific regions of the basal ganglia, with 51-82% loss in the globus pallidus, and 89-98% loss in the caudate and putamen, respectively (Rajput et al., 2008). In early stages of the disease, motor symptoms respond well to treatment with the DA precursor, 3,4-dihydroxy-L-phenylalanine or levodopa (L-DOPA), but the effectiveness of DA replacement eventually wanes in many patients. Surviving neurons in PD are also characterized by the presence of Lewy bodies (reviewed in Samii et al., 2004), suggestive of ongoing protein aggregation.

Compared to other brain regions, the SN is interesting in that the SN DAergic neurons are lost normally with age, with up to 50% loss by age 70 (McGeer et al., 1977). It has been suggested that neurons can compensate for loss by increasing DA production and turnover in the remaining neurons, or promoting neurite outgrowth (Fornstedt et al., 1990). Whether these compensatory mechanisms are protective against neuronal deficits or contributing to neuronal loss is not known. Nevertheless, there is a threshold of DA and dopaminergic neuron loss that leads to the motor symptoms of PD.

1.1.2 Idiopathic PD

Most PD cases are idiopathic, and are more or less similar in pathological and clinical characteristics from patient to patient. Such similarity in symptomology and pathology suggests a common mechanism, though no one initiator or risk factor has been identified. Thus it is believed that the cause may be due to genetic or environmental factors, or a combination of the two (Thomas and Beal, 2007). Toxic exposures, including industrial chemical exposure, exposure to heavy metals, and pesticide and herbicide exposure from farming, in well water, or living in a rural environment have all been suggested to increase PD risk (Gorell et al., 1997; Kamel et al., 2007; Olanow and Tatton, 1999; Powers et al., 2003; Powers et al., 2006; Powers et al., 2008). Genetic links have also been suggested to contribute to sporadic PD risk, including polymorphisms in mitochondrial DNA (mtDNA) and mitochondrial respiration enzymes (Sheehan et al., 1997; Swerdlow et al., 1996; Trimmer et al., 2004; van der Walt et al., 2003). Despite years of study, however, the mechanism initiating degeneration in PD is still unknown, and no strong connection between any of the above factors and idiopathic PD has been established. However, a number of genes have been identified in connection with rare familial forms of PD. Study of these familial forms of PD is providing insights to the potential mechanisms of the disease.

1.1.3 Familial Forms of PD and What They Teach Us

Familial forms of PD linked to single gene mutations have been identified, but are estimated to make up only 10% or less of total PD cases (Thomas and Beal, 2007). Nevertheless, these rare genetic mutations give us a glimpse into the underpinnings of PD

pathogenesis. Multiple gene loci have been associated with familial forms of PD, which are designated as PARK1-PARK13. Of these, mutations in specific genes have been identified for nine, including the autosomal-dominant inheritance associated with *alpha-synuclein* (PARK1 and 4), *UCH-L1* (PARK5), and *LRRK2* (PARK8); autosomal recessive inheritance associated with *parkin* (PARK2), *PINK1* (PARK6), *DJ-1* (PARK7) and *ATP13A2* (PARK9); and increased risk associated with mutations in *Omi/HTRA2* (PARK13) (reviewed in Thomas and Beal, 2007). Numerous studies are beginning to decipher the roles for gene mutations in PD pathogenesis, and have provided a wealth of information on potential mechanisms in the etiology of PD. Interestingly, many of these genes and their resulting proteins link mitochondria and oxidative stress with PD, two factors strongly associated with PD pathogenesis (Thomas and Beal, 2007). These include the proteins alpha-synuclein, parkin, PINK1, DJ-1, and Omi/HtrA2.

Alpha-Synuclein - Oxidative stress, Aggregation, and Mitochondrial instability

Alpha-synuclein is a presynaptic protein of unknown function, though it has been associated with several cellular processes, including roles in vesicle storage and recycling (Abeliovich et al., 2000; Yavich et al., 2004), as a chaperone (Ahn et al., 2006; Kim et al., 2000; Ostrerova et al., 1999; Souza et al., 2000), and in regulating DA biosynthesis (Perez et al., 2002; Tehranian et al., 2006). Mutations in the alpha-synuclein gene were the first direct genetic links to PD discovered (Polymeropoulos et al., 1997) and are thought to account for only a small portion of familial PD cases (Vaughan et al., 1998; Wang et al., 1998). Evidence indicates that alpha-synuclein is prone to aggregation, which was found to be augmented by the genetic variations associated with PD, and by oxidative and nitrative modifications (Conway et al., 1998; Giasson et al., 2000; Giasson et al., 2001; Paxinou et al., 2001). Alpha-synuclein aggregates

form fibrillar deposits, which are major components of Lewy bodies and Lewy neurites, pathogenic hallmarks of PD (Baba et al., 1998; Spillantini et al., 1998). Mice overexpressing a A53T mutant form of human alpha-synuclein also displayed mitochondrial abnormalities associated with aberrant aggregation of alpha-synuclein in brainstem and spinal cord neurons (Martin et al., 2006). Overexpression of mutant alpha-synuclein also increased the sensitivity of mice to mitochondrial toxins, such as 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP), while mice lacking alpha-synuclein were more resistant to mitochondrial toxins (Dauer et al., 2002; Klivenyi et al., 2006; Nieto et al., 2006). Thus, while sensitive to oxidative stress and aggregation, aberrant accumulation of alpha-synuclein also appears to play a role in mitochondrial stability, linking these factors together in association with PD pathogenesis.

DJ-1 – Antioxidant Function and Mitochondrial Localization

While the specific function of the ubiquitous, conserved protein DJ-1 remains unknown, various studies have implicated this protein in an oxidative stress response and antioxidant mechanisms. The antioxidant properties of DJ-1 may be attributed to the protein's own susceptibility to oxidation (Wilson et al., 2003). In cell culture, DJ-1 prevented H₂O₂-induced toxicity by oxidizing itself as indicated by a shift in the isoelectric point of the protein (Taira et al., 2004). Further study revealed that the residue cys-106 is selectively modified to a cysteinyl sulfinic acid in the oxidized form of DJ-1 (Canet-Aviles et al., 2004). In addition, siRNA-induced downregulation of DJ-1 expression or expression of mutant DJ-1 resulted in an increased susceptibility to H₂O₂ and ER stress in cells (Takahashi-Niki et al., 2004; Yokota et al., 2003). Thus DJ-1 appears to be a strong candidate as an antioxidant protector. Though primarily cytosolic, DJ-1 has also been shown to localize within the matrix and intermembrane

space of the mitochondria in mouse brain (Zhang et al., 2005), and associate with the mitochondrial chaperone mortalin/GRP75/mtHSP70 (Li et al., 2005), though with unknown function. It has also been demonstrated in cell culture that following oxidative stress, cytosolic DJ-1 translocated to the mitochondria (Lev et al., 2008), though the mechanism involved or the function of DJ-1 once in the mitochondria is unknown. It has been hypothesized that DJ-1 serves as a sensor of oxidative stress, and thus may respond to mitochondrial-generated ROS in times of mitochondrial distress in order to protect against further oxidative damage (Dodson and Guo, 2007).

Parkin, PINK1, and Omi/HtrA2

Parkin is an E3 ubiquitin ligase protein that plays a role in the ubiquitin-dependent proteasome pathway, and mutations in parkin have been associated with dysfunction in the protein degradation pathway (Kitada et al., 1998; Shimura et al., 2000; Zhang et al., 2000). It was previously hypothesized that parkin mutations exhibited their toxic effects through deficiencies in the proteasome pathway (Kahle and Haass, 2004). However, studies suggest that parkin also has proteasome-independent roles affecting multiple cellular functions (Dodson and Guo, 2007; Thomas and Beal, 2007). Parkin loss-of-function mutants in *Drosophila* exhibited increased sensitivity to oxygen radical stress and severe mitochondrial damage in muscle and germline tissues that included swollen mitochondria and fragmented cristae (Greene et al., 2003; Pesah et al., 2004), suggesting a role for parkin in mitochondrial stability. Further, mitochondrial respiratory defects have been noted in brains of knockout mice, in association with oxidative stress (Palacino et al., 2004), and in leukocytes from PD patients with parkin mutations (Muftuoglu et al., 2004). Recent studies have strengthened the role of parkin in mitochondrial

function through its association with the PD-related mitochondrial protein PINK1 (Clark et al., 2006; Park et al., 2006a).

The PTEN-induced putative kinase 1 protein, PINK1, is a nuclear-expressed mitochondrial serine protease, directly linking a mitochondrial protein with PD pathogenesis. Little is known about the function of PINK1. Valente et al. and others hypothesized that PINK1 may be responsible for phosphorylation of mitochondrial proteins in times of cellular stress, and may have a role in protein stability (Beilina et al., 2005; Leutenegger et al., 2006; Valente et al., 2004). Loss-of-function PINK1 mutant *Drosophila* exhibited increased susceptibility to stress, decreased cellular ATP levels, reduced mtDNA content and mitochondrial morphological defects (Clark et al., 2006; Park et al., 2006a; Yang et al., 2006). Interestingly, the mitochondrial morphologies and tissues in which they were exhibited were noted to be remarkably similar to those of parkin mutant *Drosophila*. Studies showed that parkin overexpression completely rescued the effect of the loss of PINK1, but not the other way around (Clark et al., 2006; Park et al., 2006a; Yang et al., 2006), while double knockouts exhibited the same level of deficits as either model alone (Clark et al., 2006; Park et al., 2006a). These studies suggest that the PD related proteins parkin and PINK1 participate in a related pathway to effect mitochondrial function and stability, in which parkin is downstream of PINK1 (Dodson and Guo, 2007).

A recent study also found that PINK1 participated in a pathway with and directly interacted with Omi/HtrA2 (Plun-Favreau et al., 2007). Omi/HtrA2 is a mitochondrial serine protease. The function of the protein is controversial. Previous studies suggested a role for the protein in promoting apoptosis following release from the mitochondria (Hegde et al., 2002; Martins et al., 2002; Suzuki et al., 2001; Verhagen et al., 2002; Yang et al., 2003). Later studies in two mouse lines, Omi/HtrA2 knockout mice and mice expressing a protease-inactive mutant

form Omi/HtrA2, found that both demonstrated premature death associated with parkinsonian-like neurodegeneration (Jones et al., 2003; Martins et al., 2004). This suggested a possible role within the mitochondria for the serine protease activity. Further, two mutations in Omi/HtrA2, which also impact serine protease activity, have now been associated with increased risk for PD (Strauss et al., 2005). Recently, Omi/HtrA2 was found to be phosphorylated at a site adjacent to a site found to be mutated in humans with PD following activation of the p38 stress pathway in cells (Plun-Favreau et al., 2007). Further, PINK1 was identified as a binding partner, and it was found that Omi/HTRA2 phosphorylation was decreased in brain tissue from PD patients who had PINK1 mutations (Plun-Favreau et al., 2007). Phosphorylation appeared to increase serine protease activity of Omi/HtrA2 and appeared to be crucial for Omi/HtrA2-mediated protection of mitochondria when cells were exposed to various toxins (Plun-Favreau et al., 2007). Whether Omi/HtrA2 is a target for PINK1 phosphorylation is uncertain. Nevertheless, these two PD-related proteins are intertwined, as are PINK1 and parkin, in a pathway responsible for mitochondrial stability during stress. The genetic links of PD described here suggests that oxidative stress and mitochondrial dysfunction play key roles in the pathogenesis of PD.

1.2 OXIDATIVE STRESS IN PD

Studies of PD patients and post mortem tissue combined with studies of experimental PD models have hinted at pathogenic mechanisms directly related to oxidative stress, including mitochondrial dysfunction and oxidative protein modification (Beal, 2007; Betarbet et al., 2002b; Dauer and Przedborski, 2003; Jenner, 2003; Schapira, 2008). Whether these pathways are a

cause for PD or the result of PD pathogenesis remains to be elucidated, but their involvement is apparent.

1.2.1 Definition of Oxidative Stress

The term reactive oxygen species (ROS) encompasses the family of oxygen-derived molecules, both radicals and non-radicals (Halliwell, 2006). ROS carry the potential to react with and oxidize other molecules, which can lead to oxidative damage in living cells (Halliwell, 2006; Halliwell, 2007). Oxidative stress is typically denoted by an imbalance between reactive species, typically an overabundance of ROS, and countering antioxidant defenses in the cellular environment (Halliwell, 2007). Oxidative stress has been implicated in the pathogenesis of neurodegenerative diseases, including PD (Beal, 2002; Beal, 2003; Butterfield et al., 2001a; Butterfield et al., 2001b; Butterfield and Kanski, 2001; Carri et al., 2003; Halliwell, 2001; Halliwell, 2006; Honda et al., 2004; Jenner, 2003; Jenner and Olanow, 1996)

1.2.2 Evidence for Oxidative Stress in PD

Glutathione and Antioxidant Defenses

An early and distinct sign of oxidative stress associated with PD is the significant loss of the antioxidant tripeptide glutathione (GSH), up to 40% loss, in PD SN, but not in other brain regions (Jenner et al., 1992; Pearce et al., 1997; Riederer et al., 1989; Sian et al., 1994a; Sofic et al., 1992). It is thought that this loss occurs early in the disease process, as loss of SN GSH was also found in Incidental Lewy Body disease (Dexter et al., 1994b), a presumed presymptomatic state of PD. Interestingly, there was no observed increase in oxidized GSH in PD brain (Sian et

al., 1994b; Sofic et al., 1992), nor alterations in activity of enzymes related to GSH turnover, including gamma-glutamyl cysteine synthetase, GSH reductase, or GSH transferase (Sian et al., 1994b). All of these findings suggest an oxidative environment in PD SN. Even in normal brain tissue, human SN was found to have the lowest GSH level in the brain (Perry et al., 1982). This finding led some to suggest that a combination of low GSH and high oxidative stress would contribute to PD (Cohen, 1983; Perry et al., 1982). It has also been suggested that reduced GSH levels precede the increased iron levels observed in PD (Jenner and Olanow, 1996) and thus may contribute to increased iron oxidation in PD SN.

In addition to GSH, the antioxidant α -tocopherol was found to be at its lowest in mouse SN (Fariello et al., 1987), further suggesting a typical yet increased potential for oxidative stress in SN. Tissue from PD patient brain also displayed increased superoxide dismutase (SOD) activity, suggestive of increased levels of the reactive oxygen radical superoxide (Saggu et al., 1989). SOD enzymes are responsible for the conversion of superoxide to hydrogen peroxide (H_2O_2). In accordance with this observation, elevated levels of the manganese-dependent mitochondrial form of SOD (SOD2) have been observed in the cerebrospinal fluid (CSF) of PD patients (Yoshida et al., 1994). The increases in SOD2 observed in PD may be compensatory for increased ROS production by impaired mitochondria (discussed in greater detail below). Overall, the SN appears to have a lower antioxidant defense system in normal brain, and oxidative stress is significantly elevated in association with PD, either as a consequence or mechanism of PD pathogenesis.

Iron and PD

The reduced transition metal ferrous iron (Fe^{2+}) can participate in reactions with ROS such as H_2O_2 , forming Fe^{3+} ions and the highly reactive and toxic hydroxyl radical ($\text{OH}\cdot$) (Riederer et al., 1989; Youdim et al., 1989). Iron levels are increased in PD SN (Dexter et al., 1991; Dexter et al., 1989b; Hirsch et al., 1991; Hirsch and Faucheux, 1998; Sofic et al., 1991), with an increase in the ratio of $\text{Fe}^{3+}/\text{Fe}^{2+}$ ions (Riederer et al., 1989; Sofic et al., 1991; Sofic et al., 1988). Combined with the lower antioxidant potential described above, the increased iron and resulting oxidant potential of the SN may contribute to PD pathogenesis.

Oxidative Damage to DNA, Lipids, and Proteins in PD

Increased markers of DNA, lipid, and protein oxidation have all been noted in advanced PD SN (Jenner, 2003; Jenner and Olanow, 1996), but interestingly not in Incidental Lewy Body disease SN (Alam et al., 1997a; Dexter et al., 1994b), which may suggest that these features are associated with PD degeneration specifically. Oxidative damage to DNA has been detected in many neurodegenerative disorders (Browne et al., 1997; Ferrante et al., 1997; Gabbita et al., 1998), including PD (Alam et al., 1997b; Halliwell, 2001). Of the four base pairs that comprise DNA, guanine is the most sensitive to oxidative modification, and thus far is the only base found to be oxidatively modified in PD. Increased levels of the oxidized forms of guanine have been identified in post mortem brain in the caudate, putamen, SN, and cerebral cortex of PD patients as compared to control patients (Alam et al., 1997b; Sanchez-Ramos et al., 1994). Increases in oxidized cytosolic RNA and mitochondrial DNA (mtDNA) were also found in PD patient SN (Zhang et al., 1999). In addition to further supporting the role of oxidative stress in PD, the

increased amount of DNA and mtDNA oxidation in the SN could contribute to cell death through DNA damage, such as breakage and mutation.

Unsaturated lipids of the cellular and mitochondrial membranes are also susceptible to oxidative damage, resulting in toxic byproducts. In lipid peroxidation, ROS react with unsaturated fatty acid double bonds to generate reactive lipid peroxyradicals. Peroxyradicals, in turn, react with other fatty acids leading to the formation of lipid peroxidation byproducts such as 4-hydroxy-2,3-nonenal (HNE), acrolein, malondialdehyde, and F2-isoprostanes (reviewed in Barnham et al., 2004). Signs of lipid peroxidation are evident in neurodegenerative disease, including AD (Butterfield et al., 2002; Montine et al., 2002; Montine et al., 2007) and PD (Dexter et al., 1994a; Halliwell, 2001). Levels of the peroxidation product malondialdehyde were increased in PD SN (Dexter et al., 1989a), along with increases in fatty acid hydroperoxides and cholesterol lipid hydroperoxides, other markers of lipid peroxidation (Dexter et al., 1994a) as compared to control brain. Lipid peroxidation has a profound impact on membrane fluidity and permeability. Oxidative lipid damage can lead to dysfunction of membrane-associated ion channels, modulation of membrane receptors, and structural alterations in membranous organelles (Farooqui and Horrocks, 1998). Byproducts of lipid peroxidation can also negatively impact other cellular components. Proteins are particularly susceptible to oxidation or conjugation by 4-hydroxynonenal (4-HNE) and 4-oxynonenal (4-ONE). 4-HNE and 4-ONE can adduct with nucleophilic residues such as cysteine, and have been demonstrated to modify and inactivate many proteins. Increased HNE-protein conjugates have been observed in PD midbrain (Yoritaka et al., 1996).

Indicators of protein oxidative damage have been well documented in PD (reviewed in Beal, 2002; Halliwell, 2001; Jenner, 2003). The detection of protein carbonyl formation is the

most common measure of protein oxidation. Protein carbonyls were increased in post mortem tissue in multiple regions of PD brain, including the SN, basal ganglia, cortex, globus pallidus, and cerebellum (Alam et al., 1997a; Floor and Wetzel, 1998). There is also evidence for protein damage by reactive nitrogen species (RNS) in PD. Increases in CSF nitrate levels and brain nitrosyl adducts were associated with PD (Good et al., 1998). Additionally, nitrotyrosine immunoreactivity was associated with Lewy bodies, suggesting protein oxidation by peroxynitrite (Giasson et al., 2000). Increased amounts of nitrated SOD2 have been found in PD CSF (Aoyama et al., 2000), which may indicate protein damage and inactivation. Oxidative modifications to proteins can have a deleterious impact on protein function, ultimately affecting cellular health and promoting protein aggregation as observed in association with PD pathogenesis.

1.3 MITOCHONDRIAL DYSFUNCTION IN PD

Mitochondrial structure, function, and signaling have long been implicated in neuronal aging, neuronal injury, and neurodegenerative disease (Beal, 2007; Friberg and Wieloch, 2002; Kwong et al., 2006; Murphy et al., 1999; Schapira, 2008; Toescu et al., 2000). A well-characterized phenomenon of mitochondrial dysfunction associated with neuronal distress is the formation of the mitochondrial permeability transition pore (PTP) (Friberg and Wieloch, 2002; Kim et al., 2003; Sullivan et al., 2005). The PTP is a nonselective pore formed by a complex of mitochondrial proteins between the inner and outer mitochondrial membrane allowing passage of ions and molecules <1.5 kDa (Beutner et al., 1998; Brdiczka et al., 1998; Vyssokikh and Brdiczka, 2003; Woodfield et al., 1998). Opening of the PTP is triggered by excess Ca^{2+} and

oxidative stress, and dramatically decreases the membrane potential of the mitochondria. If prolonged, PTP can lead to a loss of mitochondrial function and the release of apoptotic factors (reviewed in Olanow and Tatton, 1999; Vyssokikh and Brdiczka, 2003). PTP opening has been linked to induction of cell death pathways (Friberg et al., 1998; Grimm and Brdiczka, 2007; Halestrap et al., 1998; Lemasters et al., 1998; Tsujimoto and Shimizu, 2007).

Friberg et al. found that mitochondria from different regions of the brain (cortex, hippocampus, and cerebellum) were differentially sensitive to Ca^{2+} -induced PTP opening, and the level of sensitivity correlated with the vulnerability of these regions to damage from ischemia (hippocampus>cortex>cerebellum) (Friberg et al., 1999). More recently, Brown et al. found that while synaptic and non-synaptic mitochondria isolated from rat cerebral cortex did not differ in basal respiration or Ca^{2+} storage, synaptic mitochondria were more sensitive to Ca^{2+} induced PTP opening (Brown et al., 2006). The findings of these studies suggest that a differential susceptibility of brain regions, and even synapses and cell bodies, to injury or degeneration may correlate to the vulnerability of the mitochondria associated with those regions. This is particularly significant for PD, in which the neurological deficits in movement are associated with degeneration of axon terminals and cell bodies of the nigrostriatal pathway.

1.3.1 Evidence for Mitochondrial Dysfunction in PD

In 1989, Schapira and colleagues first identified a deficiency in mitochondrial NADH dehydrogenase (Complex I) activity associated with PD SN tissue (Schapira et al., 1989), with an average loss in activity of approximately 35% (Mann et al., 1994; Schapira, 2006). A deficiency was not found in other mitochondrial electron transport complexes, or with other brain regions in PD (Schapira et al., 1990). Studies identified a similar Complex I deficit in platelets (-20 to -

25% activity) (Blandini et al., 1998; Haas et al., 1995; Krige et al., 1992; Parker et al., 1989; Schapira, 2006), lymphocytes (Barroso et al., 1993; Yoshino et al., 1992), and, less consistently, in muscle tissue (Penn et al., 1995; Schapira, 2006; Taylor et al., 1994) from PD patients. These results suggest there is a systemic, low-grade inhibition of Complex I activity associated with PD.

Several lines of evidence also suggest a possible genetic link to Complex I dysfunction in PD. Cybrid cell lines with normal nuclear genomes but mtDNA from PD patients exhibit a Complex I deficit, a higher sensitivity to 1-methyl-4-phenylpyridinium (MPP⁺; the toxic metabolite of MPTP), and generation of Lewy body-like inclusions, suggesting that possible genetic defects in mtDNA genes encoding Complex I subunits are associated with PD pathogenesis (Sheehan et al., 1997; Swerdlow et al., 1996; Trimmer et al., 2004). In contrast, a study examining single-nucleotide polymorphisms in mtDNA comparing PD and control patients led to the discovery of a polymorphism in a gene encoding a subunit of Complex I that associated with a reduced risk of PD (van der Walt et al., 2003). The polymorphism, associated with the NADH dehydrogenase 3 subunit of Complex I, resulted in an amino acid change from threonine to alanine, and was associated with a significantly lower susceptibility to PD (van der Walt et al., 2003). While this suggests a protective effect of the polymorphism, the mechanism is not known. However, the connection of mitochondrial dysfunction to sporadic PD provides evidence for a possible underlying mechanism of pathogenesis. This association is supported by the identification of familial forms of PD that involve mitochondrial proteins, such as PINK-1, DJ-1, and Omi/HtrA2. Mitochondrial protein expression and/or abundance have also been found to be altered in PD. A recent proteomic analysis of mitochondria-enriched fractions from post mortem PD SN revealed differential expression of multiple mitochondrial proteins in PD brain as

compared to control, including subunits of Complex I, mitochondrial creatine kinase (MtCK), and the chaperone mortalin/GRP75/mtHSP70 (Jin et al., 2006). Also, decreased immunostaining for mitochondrial alpha-ketoglutarate, a protein of the tricarboxylic acid cycle, was noted in post mortem PD brain (Mizuno et al., 1994). Together, these findings support a role for mitochondrial function in PD pathogenesis (Schapira, 2006).

The specific impact of a slight, systemic decrease in Complex I activity on PD pathogenesis is not known. Evidence from studies utilizing Complex I inhibitors, such as the toxin rotenone, suggest ROS generation plays a role in Complex I deficiency-related pathogenesis (Sherer et al., 2003a), as is discussed in greater detail below. Interestingly, the loss of GSH noted in Incidental Lewy Body disease, as discussed above, is coincident with a slight Complex I deficit (Dexter et al., 1994b), further suggesting a relationship between oxidative stress and mitochondrial dysfunction in disease pathogenesis.

1.3.2 Mitochondrial Dysfunction and Oxidative Stress

The mitochondrial electron transport chain (ETC) is a known source of ROS generation and potential source of oxidative stress in cells (reviewed in Fiskum et al., 2003; Lenaz et al., 2002; Turrens, 2003; Votyakova and Reynolds, 2001)]. Mitochondrial ETC Complexes I and III are associated with formation of the ROS free radical superoxide, and inhibition of these complexes can increase production of free radicals (reviewed in Fiskum et al., 2003; Orth and Schapira, 2002). Superoxide is typically converted by SOD to H_2O_2 , which may go on to generate the highly reactive and toxic hydroxyl radical. Superoxide may also react with nitric oxide, forming reactive peroxynitrite (Halliwell, 1992; Halliwell, 2006). Production of and increases in these reactive species could ultimately lead to oxidative stress and damage in the

cellular environment (Halliwell, 2007). Both oxidative and nitrative species have been shown to inhibit complexes in the ETC (reviewed in Halliwell, 2001; reviewed in Heales et al., 1999; Nulton-Persson and Szweda, 2001). In addition, inhibition of Complex I has been demonstrated to facilitate the generation of ROS *in vitro* and potentially contribute to oxidative stress (Pitkanen and Robinson, 1996; Votyakova and Reynolds, 2001).

Critical mitochondrial proteins and components aside from the ETC are also sensitive to the oxidative state of the mitochondrion. Activity of the enzyme alpha-ketoglutarate, decreased abundance of which is detected in PD SN (Mizuno et al., 1994), has been shown to be sensitive to the redox state of the mitochondria (Kumar et al., 2003; Nulton-Persson et al., 2003; Nulton-Persson and Szweda, 2001). Other studies have shown that creatine kinase, the enzyme responsible for maintaining ATP and creatine phosphate energy stores in the cell, is susceptible to oxidative inhibition (Koufen et al., 1999; Miura et al., 1999). In addition to proteins, both mtDNA and mitochondrial lipid membranes are susceptible to age- and disease-related oxidative damage (Barnham et al., 2004; Zhang et al., 1999). Damage to either has severe effects on mitochondrial function, altering protein expression and membrane fluidity. Mitochondrial membrane fluidity and structure, in turn, may be a crucial factor in ETC function and mitochondrial function in general (Mannella et al., 2001).

1.3.3 Mitochondrial Dysfunction and Oxidative Stress as Models of PD

Mitochondrial dysfunction and associated oxidative stress are not only linked to PD pathogenesis, but intertwined themselves (reviewed in Beal, 2003; Lenaz et al., 2002). Models of PD exemplify this connection by utilizing toxins that cause mitochondrial dysfunction and

generate ROS; specifically the pesticide and Complex I inhibitor, rotenone, and the parkinsonian syndrome-inducing toxin MPTP (Dauer and Przedborski, 2003; Sherer et al., 2003a).

Rotenone

The pesticide rotenone is a lipophilic, high-affinity Complex I inhibitor (Degli Esposti, 1998). It is a naturally occurring compound that can be extracted from the roots of certain tropical plants belonging to the genus *Lonchocarpus* or *Derris*. Rotenone has been used as a potent insecticide and fish kill toxin. As discussed previously, exposure to pesticides, such as rotenone, is considered a risk factor for developing PD (Kamel et al., 2007).

Due to the lipophilic nature of the toxin, rotenone can easily gain access to all organs and tissues of the body, as well as cross the blood-brain barrier (Talpade et al., 2000). It has been demonstrated that chronic administration of rotenone to rats leads to a systemic inhibition of Complex I, and results in neurodegeneration and behavioral deficits characteristic of PD (Alam and Schmidt, 2002; Betarbet et al., 2000; Fleming et al., 2004; Sherer et al., 2003a; Sherer et al., 2003b). Rotenone exposure caused selective dopaminergic degeneration in the striatum and SN of treated rats, ubiquitin- and alpha-synuclein-positive protein inclusions in SN neurons, and behavioral deficits akin to bradykinesia, rigidity, and postural deficits observed in PD (Betarbet et al., 2000; Sherer et al., 2003b). *In vivo*, oxidative damage in the form of increased protein carbonyl formation was observed to be elevated in select brain regions following rotenone, with highest increases found in the midbrain and olfactory bulb (Sherer et al., 2003a). Rotenone toxicity has also been examined in human neuroblastoma cells (Sherer et al., 2003a; Sherer et al., 2001; Watabe and Nakaki, 2007b), and organotypic cultures (Sherer et al., 2003a; Testa et al., 2005). Increased protein carbonyls and decreased GSH (-57%) levels were observed following

chronic rotenone exposure in SK-N-MC cells, while organotypic cultures displayed increased carbonyl formation and loss of dopaminergic neuron projections (Sherer et al., 2003a; Testa et al., 2005). Treatment with the antioxidants alpha-tocopherol and coenzyme-Q 10 also protected against rotenone toxicity and oxidative damage in cell culture (Sherer et al., 2003a; Testa et al., 2005), suggesting that rotenone toxicity results from increased oxidative stress.

To determine whether the rotenone-induced toxicity and ROS generation was the consequence of rotenone's action on mitochondrial complex I, studies examined the effect of expressing a rotenone insensitive NADH dehydrogenase enzyme, Ndi1, *in vitro* and *in vivo*. The neurotoxic effects associated with rotenone exposure were blocked in both SK-N-MC cells transfected with Ndi1 (Sherer et al., 2003a) and in rats expressing Ndi1 via adenovirus-mediated delivery in to the SN (Marella et al., 2008). These studies demonstrate that the toxic effects of rotenone are mediated by its ability to inhibit complex I.

There is a clear association between complex I inhibition and ROS generation in the rotenone model of PD, which resembles the complex I deficiency observed in idiopathic PD. Additional studies will be necessary to determine whether rotenone toxicity can also model the non-dopaminergic degeneration and non-motor deficits associated with PD. Equally important is elucidating the mechanisms behind the selective degeneration of SN dopaminergic neurons, despite the systemic effect of the toxin. There is evidence that endogenous DA may play a role in and even potentiate rotenone toxicity in PC12 cells (Dukes et al., 2005; Liu et al., 2005). In addition, rotenone toxicity was increased in SH-SY5Y human neuroblastoma cells when intracellular levels of DA were elevated by inhibiting DA metabolism (Watabe and Nakaki, 2007b). Rotenone-induced toxicity was decreased when DA synthesis was inhibited (Watabe and Nakaki, 2007b). Rotenone-generated ROS was also associated with a redistribution of DA from

vesicles to the cytoplasm (Watabe and Nakaki, 2007b). Together, these studies suggest rotenone may have a differential impact on dopaminergic cells due to their endogenous neurotransmitter. Thus, the similarities to PD in pathological and behavioral characteristics generated via mitochondrial complex I inhibition support rotenone as a useful model in studying the mechanisms of PD.

MPTP/MPP+

The chemical MPTP is a dopaminergic toxin discovered accidentally in the early 1980's. An impurity resulting from the illicit production of a meperidine analog (1-methyl-4-phenyl-4-propionoxypiperidine; MPPP or "synthetic heroin"), the MPTP contaminant taken intravenously with the intended drug caused chronic, idiopathic parkinsonian symptoms in exposed young drug addicts (Langston et al., 1983; Langston and Ballard, 1983) and lesioning of the SN (Davis et al., 1979; Langston et al., 1999). Since the discovery, MPTP has been a widely used and very well characterized model of PD, and is most typically used in *in vivo* mouse and non-human primate models. The model has also been used in cell culture studies using various non-dopaminergic and dopaminergic cell lines, utilizing the MPTP metabolite MPP+ (see below). MPTP has also been shown to cause parkinsonian symptoms in non-human primates. MPTP exposed humans and non-human primates have demonstrated the loss of dopaminergic neurons and terminals in patterns similar to that of PD (reviewed in Dauer and Przedborski, 2003). However, they did not appear to develop classical Lewy bodies (Forno et al., 1993; reviewed in Dauer and Przedborski, 2003). Also, at least in non-human primates, degeneration of PD-associated non-dopaminergic neuron populations, such as the locus coeruleus, was inconsistent (Forno et al., 1993; Forno et al., 1986). It is thought that these discrepancies between PD and MPTP exposure may be

attributed to the acute exposure of MPTP toxicity in these situations (Dauer and Przedborski, 2003). Nonetheless, the MPTP model remains a widely utilized model for PD research due to the advantages that (1) it replicates L-DOPA-responsive, clinical motor symptoms almost identical to PD in humans, and (2) it is a relatively selective toxin of dopaminergic neurons in the brain (reviewed in Dauer and Przedborski, 2003).

MPTP toxicity is elicited through its metabolite MPP⁺, which is produced via metabolism by monoamine oxidase B (MAO-B) in glial cells, then oxidized. MPP⁺ is then exported by an unknown mechanism and selectively taken up by monoaminergic neurons via the DA transporter (DAT), norepinephrine transporter, and serotonin transporter (Javitch et al., 1985; Javitch and Snyder, 1984; Mayer et al., 1986), though toxic effects are most prominent in dopaminergic neurons (Dauer and Przedborski, 2003). In the cell, MPP⁺ has been found to interact with cytosolic proteins (Klaidman et al., 1993), bind the vesicular monoamine transporter (VMAT) and enter synaptic vesicles (Liu et al., 1992), and accumulate in mitochondria (Ramsay and Singer, 1986). The mitochondria appear to be the primary site of action by MPP⁺, where the compound was shown to act as a Complex I inhibitor (Mizuno et al., 1987; Nicklas et al., 1985; Ramsay et al., 1986). MPTP administration in mice resulted in a loss of ATP in striatal and ventral midbrain regions (Chan et al., 1991) and increased production of ROS, including the hydroxyl radical (Adams et al., 1993; Rossetti et al., 1988; Smith and Bennett, 1997). MPTP-treated mice also demonstrated a loss of GSH (Oishi et al., 1993). The loss of ATP and increased oxidative damage are cited as the primary factors in MPTP-induced cell death. In accord with this hypothesis, MPTP toxicity was attenuated *in vivo* by antioxidant administration (Park et al., 2004), overexpression of the cytosolic ROS scavenger SOD1

(Przedborski et al., 1992), and by agents improving mitochondrial energy production, such as coenzyme-Q 10 and nicotinamide (Schulz et al., 1995).

DA itself may also play a critical role in MPTP toxicity, as suggested by the selective sensitivity of dopaminergic neurons to MPTP neurodegeneration compared to other monoaminergic populations (Dauer and Przedborski, 2003). The selectivity of dopaminergic neurons to MPTP-induced toxicity appears to be due to uptake by DAT, as studies have found that chemically blocking or knocking out expression of DAT eliminated MPTP-induced toxicity (Bezard et al., 1999; Javitch et al., 1985). Once in the cells, MPP⁺ can be taken up by VMAT. Sequestering MPP⁺ in vesicles, via VMAT, is in part considered a protective mechanism. Cells overexpressing VMAT2 were found to be resistant to MPP⁺ toxicity (Liu et al., 1992), while VMAT2-null mice had enhanced MPTP-induced toxicity (Takahashi et al., 1997). However, a study by Lotharius and O'Malley found that MPP⁺ displaces DA from vesicles in mesencephalic culture, leading to further oxidation and ROS generation (Lotharius and O'Malley, 2000). Increased oxidation products of DA, specifically cysteine-bound DA (discussed below), are also observed in MPTP treated mice (Teismann et al., 2003a).

1.4 DA OXIDATION AND PD

As mentioned above, multiple brain regions are now known to be associated with PD pathogenesis, particularly in mid- and late-stage disease (Braak et al., 2003; Braak et al., 2004). However, the pronounced loss of nigrostriatal dopaminergic neurons of the SN, along with decreases in striatal DA and DA terminals, remain the primary characteristics of disease progression, and are believed to be the major contributors to the movement disorders associated

with PD (Kish et al., 1988; Rajput et al., 2008). Because the degeneration of dopaminergic neurons is clearly linked to oxidative stress, the potentially reactive neurotransmitter DA may be a contributing factor to PD pathogenesis. (Greenamyre and Hastings, 2004; Hastings and Berman, 2000; Stokes et al., 1999).

1.4.1 DA Oxidation, Protein Modification, and PD

The DA molecule is comprised of a catechol ring and an ethylamine side chain, a structure that leaves DA vulnerable to oxidation even at physiological pH (Graham, 1978). Normally, DA is stably stored at high concentrations and reduced pH within vesicles (Schuldiner, 1994; Sulzer and Rayport, 1990). However, if not adequately sequestered, DA is susceptible to oxidation through two separate pathways, (1) metabolism and (2) auto- or enzymatic oxidation. The metabolism of the ethylamine side chain of DA by MAO leads to the formation of an aldehyde metabolite, 3,4-dihydroxyphenylacetaldehyde (DOPAL) via deamination (Maker et al., 1981). The aldehyde is quickly oxidized by aldehyde dehydrogenase to form 3,4-dihydroxyphenylacetic acid (DOPAC), and H_2O_2 is formed as a byproduct (Cooper et al., 1991; Florang et al., 2007) (**Figure 1**).

DA, as well as the precursor DOPA and metabolite DOPAC, can also be oxidized. The catechol ring structure of DA will readily oxidize to form the DA quinone, producing superoxide and H_2O_2 , respectively (**Figure 1**) (Bindoli et al., 1992; Hastings and Berman, 2000; Monks et al., 1992). This can happen through auto-oxidation, which may be accelerated by the presence of iron and other transition metal ions (Graham, 1978; Miller et al., 1990), or in the presence of other oxidants such as nitrite and peroxynitrite (LaVoie and Hastings, 1999). DA quinones may also be generated through enzymatic oxidation of the catechol ring by enzymes including

prostaglandin H synthase (cyclooxygenases), tyrosinase, lipoxygenase, and xanthine oxidase (Foppoli et al., 1997; Hastings, 1995; Korytowski et al., 1987; Mattammal et al., 1995; Rosei et al., 1994). Expression and activity of these enzymes has been noted in the brain (Miranda et al., 1984; Naidu et al., 1992; Teismann et al., 2003b; Tief et al., 1998; Wajner and Harkness, 1989; Zhang et al., 2006), though the expression of tyrosinase protein in dopaminergic neurons is controversial (Greggio et al., 2005; Ikemoto et al., 1998; Tribl et al., 2007; Xu et al., 1997).

The DA quinone is an electron deficient molecule open to nucleophilic attack. An abundant and reactive biological nucleophile is the sulfhydryl group, which has readily accessible electron pairs at physiological pH (Graham et al., 1978; Monks et al., 1992; Tse et al., 1976). DA quinones react rapidly with and bind to sulfhydryl groups on free cysteine, glutathione, and protein cysteinyl residues in the cell, resulting in formation of 5-cysteinyl-DA conjugates (**Figure 1**) (Hastings et al., 1996; Ito and Fujita, 1982; Ito et al., 1988; Spencer et al., 1998; Tse et al., 1976; Zhang and Dryhurst, 1994). As many vital proteins contain active-site cysteine residues whose oxidation states are critical for function, modification by DA quinone may have detrimental effects on the health of the cell, thus contributing to the toxic potential of DA and DA quinone.

In vitro exposure to DA and DA quinones has been demonstrated to modify the structures and functions of several proteins relevant to dopaminergic and neuronal cell function. DA oxidation inhibited the activities of the DA transporter and glutamate transporter in isolated rat striatal synapses (Berman and Hastings, 1997; Berman et al., 1996). Tyrosine hydroxylase, the rate-limiting enzyme in catecholamine biosynthesis, was also found to be modified following *in vitro* exposure to DA quinone, which covalently binds and inactivates the protein, and potentially alters it to a redox-cycling quino-protein (Kuhn et al., 1999; Xu et al., 1998). Cytosolic and

mitochondrial forms of creatine kinase, a cysteine-dependent enzyme critical in maintaining ATP levels, can also be inhibited by exposure to DA and DA oxidation, (Jiang et al., 2002; Maker et al., 1986; Miura et al., 1999) potentially contributing to bioenergetic defects in the cell. Alpha-synuclein aggregation and oxidative DA modification have also been linked, showing that covalent modification by DA stabilized and promoted the aggregation of alpha-synuclein protofibrils, thought to be pathogenic, and prevented assembly of mature fibrils (Conway et al., 2001; Rochet et al., 2004). Norris et al. later found that while DA oxidation was necessary for this to occur, covalent modification of alpha-synuclein by DA was not, as DA oxidation by-products interacted with alpha-synuclein to induce conformational changes that prevented mature fibril formation (Norris et al., 2005). These studies suggest a relationship between oxidative stress, DA, and alpha-synuclein that could lead to the promotion of PD pathogenesis (Rochet et al., 2004). Exposure to oxidized DA was also shown to cause tau protein to polymerize (Santa-Maria et al., 2005a) and neurofilament proteins to covalently crosslink (Montine et al., 1995), while actin was found to bind DA in a Fe^{2+} -mediated process (Velez Pardo et al., 1995). These findings suggest DA oxidation may also have an impact on cellular cytoskeletal integrity.

There is substantial evidence of DA oxidation and DA modification of thiols occurring *in vivo*. Neuromelanin, the dark pigment found in dopaminergic cells of the SN, is ultimately formed from the polymerized products of oxidized DA, and contains oxidized forms of DA-modified cysteine (Carstam et al., 1991; Odh et al., 1994; Wakamatsu et al., 2003), demonstrating a regular occurrence of DA oxidation in pigmented SN cells. As the components of neuromelanin require DA oxidation for starting material, the formation of neuromelanin suggest a lower antioxidant defense in pigmented dopaminergic cells that would allow for DA oxidation (Zhang and Dryhurst, 1995). Interestingly, the neuromelanin-containing cells of the

substantia nigra are those preferentially lost in PD (Hirsch et al., 1988; Mann and Yates, 1983). However, neuromelanin may also have a protective role in removing excess catecholamines, oxidized DA products, and chelating harmful metals (Wakamatsu et al., 2003; Zecca et al., 2003)

Cysteiny catechols can also be identified *in vivo*. Using high-pressure liquid chromatography techniques, detectable levels of 5-cysteiny-DA, -DOPA, and -DOPAC were found in post mortem human tissue (Fornstedt et al., 1986) and were shown to be increased in PD patient SN (Spencer et al., 1998). Further, Fornstedt et al. demonstrated that 5-cysteiny catechol/catechol ratios in post mortem PD patient SN were increased as the loss of SN pigmentation increased, suggesting an enhanced rate of catechol oxidation and cysteine modification correlating with increased degeneration (Fornstedt et al., 1989). Recently, LaVoie et al. demonstrated the presence of DA-bound parkin protein from post mortem tissue of normal human SN (LaVoie et al., 2005). This finding not only demonstrates that proteins are targets for DA conjugation *in vivo*, but also provides a link between DA-induced protein modification and a specific protein associated with PD. Thus, DA oxidation occurs normally in human SN, and may be contributing to PD pathogenesis.

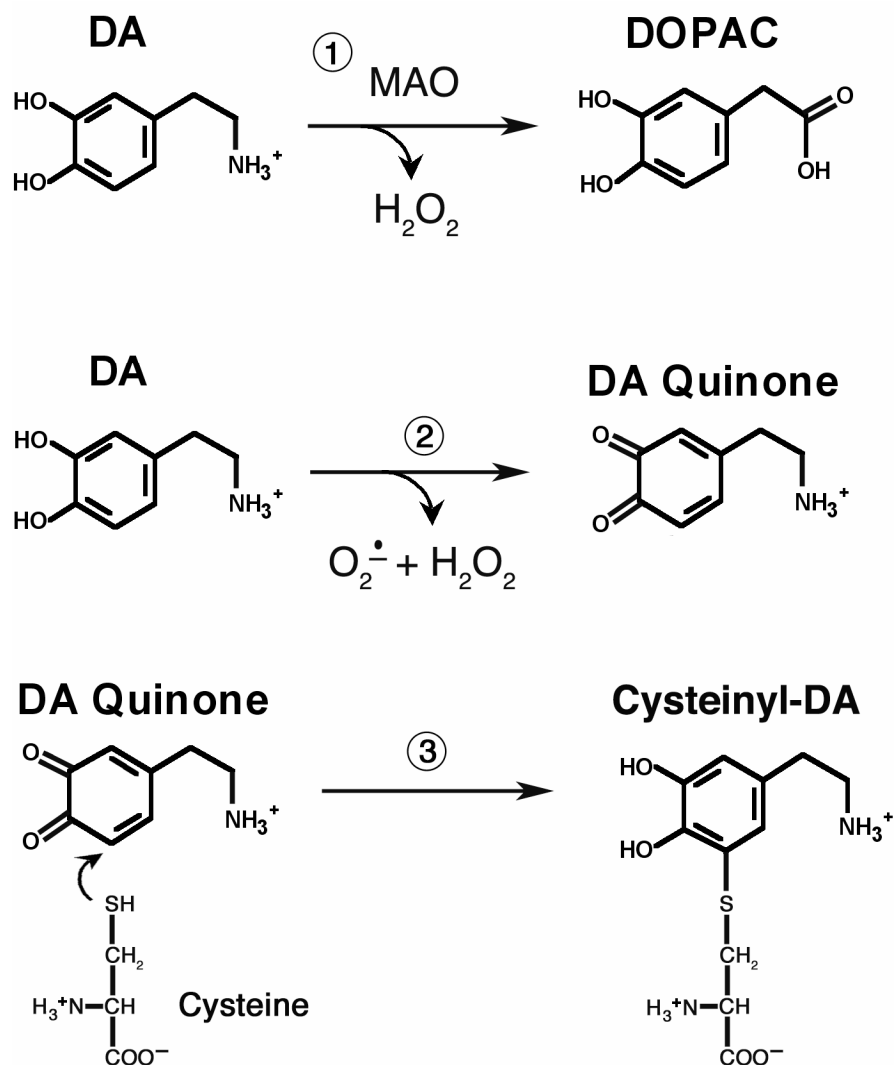


Figure 1: Metabolism and Oxidation of DA.

(1) Deamination of DA by MAO and consequent oxidation leads to the formation of DOPAC, with H_2O_2 as a byproduct. (2) DA can also undergo enzymatic- or autooxidation to form DA quinone, producing superoxide ($\text{O}_2^{\cdot -}$) and H_2O_2 . (3) The DA quinone will react with sulfhydryl groups, covalently bonding to free cysteines and protein cysteinyl residues in the cell to form a cysteinyl-DA conjugate.

1.4.2 Models of DA Oxidation and Toxicity

Both DA metabolism and DA oxidation are known to lead to ROS and free radical generation. In addition, reactive metabolites of DA can lead to protein oxidation and covalent modification, all thought to be contributors to DA-induced toxicity. Studies have taken advantage of the reactive chemistry of DA to study PD mechanisms, including 6-OHDA and DA toxicity models.

6-OHDA

6-hydroxydopamine (6-OHDA) toxicity represents the first animal model of PD, introduced over 30 years ago, and was the first agent discovered to cause a specific neurotoxic effect to dopaminergic neurons (Jonsson and Sachs, 1975; Sachs and Jonsson, 1975; Ungerstedt, 1968). Since its introduction, 6-OHDA has been extensively used both *in vivo* and *in vitro* to study mechanisms of dopaminergic neuron degeneration. 6-OHDA has chemical structure similar to DA, and is a substrate for the DAT and the norepinephrine transporter. As a result, 6-OHDA toxicity is relatively selective to monoaminergic neurons (Luthman et al., 1989). To elicit effects on dopaminergic structures of the brain, 6-OHDA must be administered centrally, as it cannot cross the blood-brain barrier (Dauer and Przedborski, 2003).

6-OHDA is thought to elicit toxicity primarily through oxidative stress and quinone formation (Cohen, 1984). Like DA, 6-OHDA has the potential to undergo autooxidation, producing H_2O_2 and the redox cycling paraquinone, producing ROS and resulting in dopaminergic cell death due to oxidative stress (Heikkila and Cohen, 1971; Kumar et al., 1995; Oiwa et al., 2003; Saner and Thoenen, 1971). Studies *in vivo* and *in vitro* have also demonstrated toxic effects of 6-OHDA, including markers of lipid peroxidation (Kumar et al.,

1995), decreased GSH and SOD (Perumal et al., 1992), and apoptosis (Bensadoun et al., 1998; Galindo et al., 2003; Marti et al., 2002), many of which are relative to PD pathology. However, the 6-OHDA model does not replicate other features of PD, in that toxicity and degeneration are acute rather than prolonged and progressive, no other brain regions are involved in degeneration, and no Lewy body formation has been observed (reviewed in Betarbet et al., 2002b; Bove et al., 2005). However, the ability of the model to simulate striatal DA depletion and neuronal loss, along with the advantage of unilateral toxicity, has led to the model's extensive use in testing antiparkinsonian agents, cell transplantation studies to recover DA levels, and neurotrophic factor- and exercise-induced promotion of dopaminergic neuron survival (Dunnett et al., 1981; Schwarting and Huston, 1996; Smith and Zigmond, 2003).

DA

The ability of DA to exhibit toxic effects in various cell culture studies is well established (Koshimura et al., 2000; Offen et al., 1995; Orth and Tabrizi, 2003). DA toxicity in cell culture has been attributed to factors including oxidative stress, protein modification, alterations in mitochondrial respiration and membrane potential, and apoptosis (Ben-Shachar et al., 2004; Brenner-Lavie et al., 2008; Jones et al., 2000; Lai and Yu, 1997a; Lai and Yu, 1997b; Offen et al., 1996; Si et al., 1998; Wang et al., 2008b). DA toxicity has also been demonstrated *in vivo*. Intrastratial injection of DA in rats results in specific loss of striatal dopaminergic nerve terminals and axons (Hastings et al., 1996; Rabinovic and Hastings, 1998; Rabinovic et al., 2000). Further, toxicity is associated with increases in cysteinyl-catechol protein modification (Hastings et al., 1996; Rabinovic and Hastings, 1998). Interestingly, no damage to DA terminals was observed following intrastratial DA administration in DAT knockout mice, suggesting that

intracellular DA oxidation is necessary for DA-induced toxicity (unpublished data from the Hastings laboratory).

While the exact mechanism of the DA-induced toxicity is not known, the toxicity of DA has been linked to oxidative stress. In several cell culture studies, antioxidants prevented DA toxicity (Hoyt et al., 1997; Passi et al., 1987). Similarly, co-administration of antioxidants with DA intrastriatal injections prevented striatal dopaminergic terminal loss and significantly decreased protein cysteinyl-catechol formation (Hastings et al., 1996). Given the ability of oxidized metabolites of DA to bind and inhibit protein function, the toxicity induced by DA is most likely more complex than simply increasing oxidative stress, and may involve modification of critical cellular proteins.

1.4.3 DA Oxidation and Mitochondrial Dysfunction

The association of oxidative stress and mitochondrial dysfunction with PD pathology has led many to study the effects of DA and DA oxidation on mitochondrial function. Exposure of isolated intact rat brain mitochondria to DA or DA quinone has been shown to alter mitochondrial respiration (Berman and Hastings, 1999; Gluck et al., 2002; Gluck and Zeevalk, 2004). Exposure of mitochondria to DA quinones also triggered opening of the PTP (Berman and Hastings, 1999). In cultured SH-SY5Y cells, exposure to DA resulted in a significant depolarization of mitochondrial membrane potential, which could be attenuated by bypassing Complex I and adding substrates for Complex II (Brenner-Lavie et al., 2008). DA also potentiated mitochondrial dysfunction induced by the Complex I inhibitor MPP⁺ in isolated rat liver mitochondria, including reduced oxygen uptake, increased swelling, and decreased membrane potential as compared to controls, and to MPP⁺ or DA alone (Boada et al., 2000),

suggesting a DA-induced effect on mitochondrial respiratory enzymes. Together the above findings suggest a modifying effect of DA or DA quinones on specific mitochondrial proteins. Though mitochondrial targets of DA oxidation have not been identified, the activities of several ETC complex proteins have been examined following DA exposure.

Rat brain mitochondrial Complex I was found to be inhibited by DA in disrupted brain mitochondria over the course of a few minutes by multiple studies, though with varying results, including 20-25% inhibition with 1-100 mM DA (Przedborski et al., 1993), 10% inhibition with 10mM DA (Morikawa et al., 1996), and 50% inhibition with approximately 12 μ M DA (Ben-Shachar et al., 2004). Kahn et al. found that extended periods of incubation (2 hr at 37 °C) with 100-400 μ M DA significantly inhibited both Complex I (20-45% inhibition) and Complex IV (30-55% inhibition) activity in disrupted rat brain mitochondria (Khan et al., 2005). Inhibition coincided with the formation of DA-bound protein, and could be blocked by adding GSH, suggesting a role for DA quinones affecting enzyme function (Khan et al., 2005). All of these studies were conducted using disrupted mitochondria, and examined only ETC protein functions. Studies have yet to confirm whether these or other proteins are subject to inhibition or covalent modification in intact brain mitochondria. However, recent studies in the Hastings and Greenamyre laboratories suggest that exposure of intact mitochondria to DAQ results in a significant decrease in Complex I activity and covalent modification of Complex I subunits by DA (Arduini et al., Society for Neuroscience Abstract 2008; unpublished data).

As previously mentioned, mitochondrial dysfunction is known to lead to ROS production. The added oxidative stress may contribute to DA oxidation, which contributes to further oxidative damage in the mitochondria and the cell. The two pathways are thus intertwined, setting up a vicious cycle of increasing oxidative damage and toxicity in the cell.

Therefore, dopaminergic neurons may be especially vulnerable to propagating oxidative stress, leading to increased cellular damage, and perhaps leading to the selective dopaminergic PD pathogenesis. Previous studies have suggested that DA oxidation leads to modifications of critical mitochondrial proteins, though no prior work has identified the specific mitochondrial protein targets of oxidized products of DA. Thus, it is of interest to identify and characterize these protein targets, as they could further elucidate pathways of neuronal injury in PD development, as well as reveal novel targets for therapeutic interventions.

2.0 THESIS GOALS

The primary goal of the work presented in this thesis was to identify mitochondrial targets of DA oxidation. DAQ can bind protein, and has been associated with the inactivation and altered aggregation of specific cellular proteins. Previous studies, including work from our laboratory, have established that mitochondrial function is altered by exposure to DA and DA oxidation products, suggesting modification of critical proteins. However, these protein targets have not previously been identified.

We utilized proteomic techniques to identify modifications to proteins following DAQ exposure of isolated rat brain mitochondria and DA exposure in cell culture. Analysis and identification of modified protein targets within the complete proteome of tissues or organelles can be accomplished using various proteomic techniques, which have previously been applied to the study of neurodegenerative disease and disease models (Butterfield et al., 2006; Johnson et al., 2005; Kim et al., 2004). Studies have identified multiple protein targets of oxidative modification and altered expression in association with multiple neurodegenerative diseases in humans, including Alzheimer's disease (Castegna et al., 2002a; Castegna et al., 2002b; Castegna et al., 2003) and Parkinson's disease (PD) (Basso et al., 2004; Jin et al., 2006). In these studies, I applied various proteomics techniques to identify targets of DA oxidation in a model of PD.

First, I utilized the two-dimensional difference in-gel electrophoresis (2-D DIGE) proteomics technique to examine changes in proteins following *in vitro* exposure of isolated rat

brain mitochondria to DAQ. 2-D DIGE allows for the comparison of two protein samples, typically control and experimental, within one gel through the use of charge- and molecular weight-matched fluorescent dyes. I utilized two sets of DIGE-compatible dyes, cysteine-reactive maleimide dyes and lysine-reactive NHS-ester dyes. I initially hypothesized that we would observe differential labeling with the cysteine-reactive dyes that would be indicative of either protein cysteinyl residues covalently modified following DAQ exposure or altered protein levels within our *in vitro* system, while differential labeling with the lysine-reactive dyes would indicate just altered protein levels. We observed, however, that both dyes indicated alterations in abundance of a subset of mitochondrial proteins. Protein alterations observed via DIGE analyses were quantified, and alterations in protein levels confirmed by Western blot. The effect of DA exposure of PC12 cells on the mitochondrial levels of two of the identified proteins, mitochondrial creatine kinase and mitofilin, was also examined. The results from these studies are discussed in **Chapter 3**.

Second, I utilized proteomics techniques, including 2-D gel electrophoresis, autoradiography, and 2-D DIGE in combination with radiolabeled DA (^{14}C -DA) to identify proteins covalently modified following exposure of isolated rat brain mitochondria to ^{14}C -DAQ. As the DIGE analyses revealed alterations in protein levels, and not covalent modifications, my goal was to identify the mitochondrial proteins that are targets of DAQ covalent binding. These proteins may further elucidate pathways in PD development, as well as reveal novel targets for therapeutic interventions. These observations are discussed in **Chapter 4**.

Lastly, I wanted to examine the effect of altered mitofilin protein levels on cellular responses to dopaminergic toxins. Mitofilin was identified in studies from both **Chapter 3** and **Chapter 4** as a target of DA-induced modifications within the mitochondria. Mitofilin was

recently shown to be important in the maintenance of mitochondrial cristae (John et al., 2005), and studies suggest that alterations in mitofilin, amongst other proteins, may play a role in neurological disorders (Myung et al., 2003; Omori et al., 2002; Wang et al., 2008a). However, no previous study has directly characterized mitofilin with regard to a neurodegenerative disease model. As exposure to DA oxidation can alter mitofilin abundance in isolated mitochondria and cell culture (see **Chapter 3**), I hypothesized that modulating the levels of mitofilin in dopaminergic cells, PC12 and SH-SY5Y, using overexpression and knock-down techniques would alter cellular responses to the toxins DA and rotenone, which can also alter mitochondrial function. The results of these studies are discussed in **Chapter 5**.

3.0 PROTEOMIC ANALYSIS OF RAT BRAIN MITOCHONDRIA FOLLOWING EXPOSURE TO DOPAMINE QUINONE: IMPLICATIONS FOR PARKINSON'S DISEASE

3.1 SUMMARY

Oxidative stress and mitochondrial dysfunction have been linked to dopaminergic neuron degeneration in Parkinson's disease. We have previously shown that dopamine oxidation leads to selective dopaminergic terminal degeneration *in vivo* and alters mitochondrial function *in vitro*. In this study, we utilized 2-D difference in-gel electrophoresis to assess changes in the mitochondrial proteome following *in vitro* exposure to reactive dopamine quinone. A subset of proteins exhibit decreased fluorescence labeling following dopamine oxidation, suggesting a rapid loss of specific proteins. Amongst these proteins are mitochondrial creatine kinase, mitofilin, mortalin, the 75 kDa subunit of NADH dehydrogenase, and superoxide dismutase 2. Western blot analyses for mitochondrial creatine kinase and mitofilin confirmed significant losses in isolated brain mitochondria exposed to dopamine quinone and PC12 cells exposed to dopamine. These results suggest that specific mitochondrial proteins are uniquely susceptible to changes in abundance following dopamine oxidation, and carry implications for mitochondrial stability in Parkinson's disease neurodegeneration.

3.2 INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra (SN) pars compacta and the formation of Lewy bodies (Samii et al., 2004). Most PD cases are considered sporadic, and provide us with limited clues to causes of disease pathogenesis. However, increasing evidence implicates mitochondrial dysfunction and oxidative stress in PD (Betarbet et al., 2002a; Dauer and Przedborski, 2003; Jenner, 2003; Pallanck and Greenamyre, 2006).

Decreased mitochondrial Complex I (NADH dehydrogenase) activity has been observed in both the SN (Janetzky et al., 1994; Orth and Schapira, 2002; Schapira et al., 1990) and periphery (Blandini et al., 1998; Shoffner et al., 1991) of PD patients. Deficiencies and inhibition of the mitochondrial electron transport chain (ETC), a known source of reactive oxygen species (ROS), can lead to increased mitochondria-generated free radicals and oxidative stress (Beal, 2003; Lenaz et al., 2002). Increased ROS may cause damage to macromolecules, such as increased oxidation of mitochondrial proteins, making them susceptible to accumulation or proteolytic degradation (Bota and Davies, 2001; Bota and Davies, 2002; Bulteau et al., 2006).

Although multiple brain regions are involved in PD, the degeneration of dopaminergic neurons under conditions of oxidative stress suggests dopamine (DA) may be contributing to PD pathogenesis (Greenamyre and Hastings, 2004; Ogawa et al., 2005; Stokes et al., 1999). Normal DA metabolism leads to the production of ROS, and DA not adequately stored in vesicles is prone to oxidation, forming the reactive DA quinone (DAQ) (Graham et al., 1978). Dopamine-induced toxicity, demonstrated both in cell culture (Jones et al., 2000; Koshimura et al., 2000) and *in vivo* (Hastings et al., 1996; Rabinovic et al., 2000), is dependent on DA oxidation and the formation of reactive DA metabolites. Post-mortem studies have found increased levels of

cysteinyln-DA, the covalent modification of cysteine by DAQ, in SN of PD patients (Fornstedt et al., 1989; Spencer et al., 1998). Dopamine and DAQ exposure also alter mitochondrial respiration (Berman and Hastings, 1999; Cohen et al., 1997; Gluck et al., 2002) and trigger permeability transition (Berman and Hastings, 1999) in isolated rat brain mitochondria, suggesting modification of critical mitochondrial proteins, though specific proteins have yet to be identified. As previous proteomic studies have identified alterations in mitochondrial proteins in animal models of PD (Jin et al., 2005; Palacino et al., 2004; Periquet et al., 2005; Poon et al., 2005b), it is of interest to identify and characterize the mitochondrial protein targets of DA oxidation. Such proteins could become therapeutic targets in PD.

In this study, we utilized 2-D DIGE techniques in combination with cysteine- and lysine-reactive fluorescent dyes as a non-biased approach to evaluate protein alterations in rat brain mitochondria immediately following *in vitro* exposure to DAQ. Differential fluorescent labeling indicated a significant loss following DAQ exposure of a subset of potentially critical proteins that were identified by subsequent mass spectrometric studies. Western blot analyses confirmed decreases in two of these proteins, mitochondrial creatine kinase and mitofilin, in isolated brain mitochondria exposed to DAQ and PC12 cells exposed to DA. These findings suggest that specific mitochondrial proteins are uniquely susceptible to oxidation-induced changes in abundance, and may have implications for PD pathogenesis.

3.3 EXPERIMENTAL PROCEDURES

Materials

Cysteine-reactive maleimide-conjugated Cy3/5 cyanine Ettan DIGE Saturation Labeling dyes (Cys-CyDyes) and lysine-reactive N-hydroxysuccinimide (NHS) ester-conjugated Cy3/5 cyanine Ettan DIGE Minimal Labeling dyes (Lys-CyDyes) were purchased from GE Healthcare (Piscataway, NJ). Sequencing Grade Modified Trypsin and Gold Mass Spectrometry Grade Modified Trypsin were purchased from Promega (Madison, WI). Solutions and stocks for in-gel trypsin digest and mass spectrometry procedures were prepared using HPLC-grade ddH₂O from Fisher Biotech (Pittsburgh, PA) and HPLC-grade MeOH and acetonitrile from Sigma-Aldrich (St. Louis, MO). Protease inhibitor cocktail (cat#P2714), DA, mushroom tyrosinase, and most general chemicals for SDS-PAGE, buffers, and solutions were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Rabbit-anti-mitochondrial creatine kinase (MtCK) and rabbit-anti-mitofilin polyclonal antibodies were generated for our laboratory by GeneMed Synthesis, Inc. (South San Francisco, CA). All other general solutions and stocks were prepared using doubly distilled water (ddH₂O) from a Milli-Q system (Millipore Corp., Bedford, MA).

Mitochondrial Isolation and Respiration

All animal procedures were approved by the Animal Care and Use Committee at the University of Pittsburgh and are in accordance with guidelines put forth by the National Institutes of Health in the *Guide for the Care and Use of Laboratory Animals*. Mitochondria-enriched fractions were isolated from the brain tissue of adult male Sprague-Dawley rats via differential centrifugation by the method of Rosenthal et al. (1987) as previously described (Berman and Hastings, 1999; Berman et al., 2000), with elimination of the protease Nagarse.

Final mitochondrial pellets were resuspended in mitochondrial isolation buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mg/ml FA-free BSA, and 1 mM EGTA, pH 7.4) and kept on ice. Mitochondrial protein content was determined by the Bradford method (1976). Prior to experimental use, respiration rates based on oxygen consumption were measured in the mitochondrial preparations to ensure mitochondrial health, as previously described (Berman and Hastings, 1999). Mitochondrial health was determined by the ratio of respiration active state 3, induced by the addition of ADP, to resting state 4, induced by the addition of oligomycin. Only mitochondria with a coupled state 3/state 4 ratio above 6 were used for this study.

Exposure of Isolated Mitochondria to Dopamine Quinone

Mitochondria (2 mg total protein) were exposed to DA (150 μ M) and tyrosinase (150 U) in modified mitochondrial isolation buffer with 25 mM HEPES minus BSA, pH 7.4 (reaction buffer) plus protease inhibitor cocktail (2.5 μ l/mg protein; Sigma) for 15 min at room temperature (RT). Following incubation, mitochondria were placed on ice and immediately pelleted by centrifugation at 15,000 x g for 15 min at 3°C. Control mitochondria underwent the same procedure in the absence of DA. Pelleted mitochondria were lysed by rigorous pipetting in denaturing 2-D DIGE lysis buffer (9 M urea, 2% w/v CHAPS, and 30 mM Tris-base, pH 8.5) in a ratio of 100 μ L buffer to 1 mg protein. Insoluble material was pelleted by centrifugation (16,000 x g for 1-2 min at RT) and discarded. Protein concentrations of lysed control and DAQ-exposed samples were determined by Bradford (1976). Thiol reducing agents were excluded from the lysis buffer to maintain proteins in a non-reduced state.

Cys- and Lys-CyDye Labeling

For cysteine-dye minimal labeling 2-D DIGE, migration-matched Cy3 and Cy5 Cys-CyDyes (GE Healthcare) were rehydrated in dimethylformamide (DMF) to a concentration of 0.5 mM, aliquoted, and stored at -20°C with desiccation until use. Prior to use, an aliquot of dye was thawed to RT and diluted in DMF to a working concentration of 62.5 μ M. Control and DAQ-exposed protein sample lysates were reacted with the indicated Cys-CyDye under non-reducing conditions at a ratio of 1 pmol dye per 2 μ g protein. We used low concentrations of Cys-CyDyes to achieve a minimal labeling effect on non-reduced protein samples. Preliminary experiments using various dye concentrations identified 1pmol dye per 2 μ g protein, which is 0.125% of the ratio utilized for saturation labeling, as optimal for minimal Cys-CyDye labeling. This concentration provided sufficient labeling for detection and imaging while maintaining reproducible results across gels (data not shown).

Samples were labeled Cys-Cy5 control and Cys-Cy3 DAQ, or the reciprocal to control for differential dye affinity. Samples were gently vortexed and incubated in the dark for 45 min at RT. The reaction was quenched by adding an equal volume amount of 2-D DIGE sample buffer (9 M urea, 2% w/v CHAPS, 2% v/v 3-10 IPG ampholyte buffer, 130 mM dithiothreitol (DTT), and a trace of bromophenol blue in ddH₂O). Final DIGE samples were prepared by combining equal amounts of Cys-CyDye labeled control protein and Cys-CyDye labeled DAQ-exposed protein.

Lysine-dye minimal-labeling 2-D DIGE analysis was utilized to control for changes in protein abundance between control and DAQ-exposure groups in comparison to Cys-CyDye DIGE. Migration-matched Cy3 and Cy5 Lys-CyDyes were rehydrated in DMF to a concentration of 0.5 mM and stored at -20°C with desiccation until use. Prior to use, dyes were

thawed to RT and diluted 1:1 in DMF. Control and DAQ-exposed protein sample lysates were reacted with the indicated Lys-CyDye (Lys-Cy5 control and Lys-Cy3 DAQ, or the reciprocal) under non-reducing conditions at a ratio of 2 pmol dye per 1µg protein in the dark for 30 min on ice. The reaction was quenched by the addition of free lysine to a final concentration of 385 µM and incubated 15 min on ice. Labeled samples were diluted 1:1 with 2-D DIGE sample buffer. Equal protein amounts of the Lys-CyDye labeled control and the DAQ-exposed samples were combined to generate a final DIGE sample for 2-D gel electrophoresis. Each DIGE gel experiment and its associated parallel gels (Cys- and Lys-CyDye DIGE gels and reciprocals) were generated from independent mitochondrial isolation and DAQ exposure experiments.

2-Dimension Difference In-Gel Electrophoresis

Samples (250 µg) were isoelectrically focused via sample cup loading on rehydrated 18 cm linear 3-10 pH Immobiline DryStrips (GE Healthcare), using a Multiphor II system with a 3501XL power supply (GE Healthcare), and using a 4-phase program with a total run of 75 kVhr. Focused strips were stored at -80°C until the second dimension run. Prior to second dimension electrophoresis, DryStrips were equilibrated at RT for 10 min in an equilibration buffer (75 mM Tris-HCl pH 6.8, 6 M urea, 30% v/v glycerol, 1% w/v SDS) supplemented with 30 mM DTT, followed by 10 min at RT in equilibration buffer supplemented with 240 mM iodoacetamide. Equilibrated DryStrips were trimmed to 13.5-15 cm and run in second dimension 12% SDS-PAGE (1.5mm thick gels, Hoefer SE600 Ruby Electrophoresis Unit).

Fluorescence Detection and Spot Picking

Immediately following the second dimension run, gels were scanned on a Typhoon 9400 scanner using ImageQuant software (GE Healthcare) to obtain a 200 μm resolution image of the gel. Following imaging, gels were fixed overnight in a 40% MeOH, 1% acetic acid solution at 4°C. The gels were scanned a second time using a fluorescent scanning automated spot picker, designed by Dr. Jonathan Minden of Carnegie Mellon University (instrumentation housed in the University of Pittsburgh Genomics and Proteomics Core Laboratories). The digital scans of the Cy3 and Cy5 dyes within each gel were compared visually with the aid of Image J imaging software (NIH). Protein spots that exhibited a noticeable change in fluorescence, and several that exhibited no change, were then picked utilizing the automated picker.

In-gel Trypsin Digest and Protein Identification

Immediately following excision from 2-D DIGE gels, gel plugs were washed with 50:50 MeOH:50 mM ammonium bicarbonate followed by dehydration in acetonitrile (ACN) and drying by speed-vacuum. The dried plugs were rehydrated with 10 μl of 20 $\mu\text{g/ml}$ trypsin in 20 mM ammonium bicarbonate and then incubated for 4 hr at 42°C. Samples were extracted by repeated washing in 1% trifluoroacetic acid in 50:50 ACN:H₂O extraction buffer, dried completely via speed-vacuum, and stored for up to 2 weeks with desiccation at 4°C.

Protein identification was completed using MALDI-TOF mass spectrometry (MS) with peptide mass fingerprinting. For MS analysis, dried samples were rehydrated (2-3 μl of 0.3% trifluoroacetic acid, 1 mM ammonium citrate in 50:50 ACN:H₂O; plus an equal volume of saturated α -cyano-4-hydroxycinnamic acid matrix solution), spotted onto a target, and mass spectra obtained using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer

(Applied Biosystems, Foster City, CA). Spectra were calibrated via external standard and internal trypsin calibration. Mass spectra retrieved from MALDI-TOF MS were processed by GPS Explorer™ (ver. 3) MS data analysis software (Applied Biosystems, Foster City, CA) coupled with Mascot™ search engine (Matrix Science) for peak list generation and database search. Criteria for peak list generation included a minimum signal to noise ratio of 5-10, with either no peak exclusions or exclusion of common trypsin peaks with a mass exclusion tolerance of 0.1 Da or 50ppm. Database search parameters included specifying a peptide mass tolerance of 50 ppm and peptide charge +1 for monoisotopic peaks, while allowing a maximum 1 missed trypsin cleavage with modifications of partial methionine oxidation, partial cysteine carboxylation, partial or complete cysteine carbamidomethylation, and/or partial cysteinyl-DA conjugation. Resulting peak lists were searched against the National Center for Biotechnology Information non-redundant (NCBIInr) database, specifying “all entries” or “*Rattus*” species. A positive protein identification for a given spot was accepted when a top ranked hit yielded a statistically significant probability-based MOWSE protein score and protein score confidence interval > 90% with a peptide count ≥ 6 , protein coverage >20%, a predicted molecular weight that was relative to the protein spot position on the gel, and could be replicated across two or more separate 2-D DIGE gel experiments.

Fluorescence Imaging and Quantitative Image Analysis

For quantitative image analysis, 2-D DIGE gels were scanned for fluorescence imaging on a Typhoon 9400 laser scanner using ImageQuant software (GE Healthcare) at 100 μm resolution using photomultiplier tube (PMT) voltage settings below saturation for each dye (Cy3/5). Settings were determined for the first set of gels, both Cys- and Lys-CyDye labeled,

then all further gels were scanned using the same PMT voltage settings, or ratio as necessary, to obtain non-saturation images. In-gel quantitative comparisons of fluorescence were completed using the Difference In-Gel Analysis (DIA) module of DeCyder Differential Analysis software (GE Healthcare). Fold change ratios, based on volume ratios of the individual spots and internally normalized by DeCyder, were determined and recorded for DeCyder-defined spots that corresponded to proteins previously identified by MS analysis. For each selected spot within a gel, the fold change was converted to percent DAQ-exposed mitochondrial protein fluorescence of control and averaged across all analyzed Cys-CyDye DIGE gels (n=6 total gels from 5 separate mitochondrial experiments) or Lys-CyDye DIGE gels (n=7 total gels from 5 separate mitochondrial experiments) using Excel (Microsoft Corp.). Images obtained from ImageQuant were prepared for presentation using Adobe Photoshop (Adobe).

PC12 Cell Culture and Mitochondrial Isolation

PC12 cells were maintained in DMEM supplemented with 7% horse serum (HS) and 7% fetal bovine serum (FBS). For differentiation, cells were subcultured on rat-tail collagen coated 100 mm plates at 1.5×10^6 cells/plate in DMEM supplemented with 1% HS, 1% FBS, and 0.1 $\mu\text{g/ml}$ NGF for 6 days. Media was then replaced with fresh differentiating media with or without 150 μM DA and cells were incubated for 16 hrs. Cells were collected by force pipetting, rinsed with PBS, and isolated by centrifugation. Mitochondrial enriched fractions were prepared from 10 confluent plates in each group using methods similar to those for isolating rat brain mitochondria, with the modification of protease inhibitor cocktail (2 $\mu\text{l/ml}$) being present in the mitochondrial isolation buffer throughout the isolation process. Mitochondria were lysed in 2-D DIGE lysis buffer and final protein concentrations were determined by Bradford (1976).

SDS-PAGE and Western Blot Immunodetection of Select Proteins

Lysed rat brain and PC12 cell mitochondrial protein samples (50 µg/lane) were run on 5-20% gradient SDS-PAGE (Hoefer ® Mighty Small II apparatus) and transferred to nitrocellulose (BioRad) for Western blot analysis via a BioRad Trans-Blot ® Semi-Dry Electrophoretic Transfer system. The membrane was removed, washed briefly in Tris-buffered saline (TBS), blocked in 0.2% w/v fat-free dry milk for 30min, rinsed briefly in TBS plus 0.1% Tween-20 (tTBS), and placed in a 1:1000 dilution of rabbit anti-MtCK or 1:5000 dilution of rabbit anti-mitofilin primary antibody in tTBS overnight at 4°C. Immunoreactive bands were visualized using the BioRad Immune-Star ® goat-anti-rabbit (dil 1:10,000) chemiluminescence detection kit and exposed to Biomax MR Film (Kodak). Mouse-anti-COXIV (dil 1:37,000; AbCam) and rabbit-anti-voltage-dependent anion channel 1 (VDAC1) (dil 1:2000; AbCam) were used as loading controls for rat brain mitochondria and for PC12 mitochondria, respectively. VDAC1 was selected because in parallel 2-D DIGE studies with PC12 cell mitochondria the protein did not significantly change following DA exposure (unpublished data). Films were digitally scanned and the densities of immunoreactive bands were determined using UN-SCAN-IT Gel (ver. 5.1) densitometry software (Silk Scientific; Orem, Utah).

Statistical Analysis

Cys- and Lys-CyDye MS-identified proteins whose relative DAQ-exposed fluorescence values (as percent of control) fell outside of a defined range of 83.3-120% (± 1.2 fold) were selected as different from control. The range represents two standard deviations in a Cy5-labeled control versus Cy3-labeled control gel analyzed by DeCyder (data not shown), and is the

recommended threshold for determining significant change in DeCyder analysis. Statistical significance was determined using a 1-sample two-tailed Z-test on the DAQ-exposed mitochondrial protein spot volume intensities expressed as percent of control, as determined from DeCyder analysis. The Z-test is optimal, as DeCyder DIA software calculates changes between corresponding control and treated protein spots within a gel as a volume ratio of the two samples, generating one value of “fold change” for each protein spot that compares both groups. The ratios are then internally normalized across the entire constellation of labeled spots. Significance for each changed DA-exposed protein from control (valued at 100% control) was determined when $p < 0.01$. The percent control values were directly calculated from the normalized DeCyder volume ratios. For Western blot analysis, rat brain mitochondria samples were run in duplicate or triplicate for each of $n=6-7$ separate experiments, and PC12 mitochondria samples were run in triplicate for each of $n=4$ separate experiments. Significance between group means was determined by two-factor ANOVA with replication followed by post-hoc Bonferroni tests.

3.4 RESULTS

3.4.1 A Subset of Proteins Exhibit Decreased Cysteine-reactive CyDye Labeling Following DAQ Exposure

Cys-Cy5 labeled control (magenta) and Cys-Cy3 labeled DAQ-exposed (cyan) mitochondrial protein lysates were compared in equal protein amounts on the same gel, revealing a reproducible pattern of protein spots (**Figure 2A**; $n=10$), within which a subset of observed

spots displayed a differential fluorescence intensity. Specifically, a subset of proteins exhibited relative decreased cysteine-reactive dye fluorescence following DAQ exposure as compared to control. Protein spots of interest, including those that exhibited an obvious change in fluorescence, were identified by peptide mass fingerprinting (**Figure 2A**). All identified proteins were determined to be mitochondria-associated proteins. A listing of all proteins identified and their corresponding data can be found in **Appendix A**.

Quantitative DeCyder analyses across a set of Cys-CyDye gels (n=6) confirmed significantly decreased fluorescence labeling of a subset of proteins in DAQ-exposed mitochondria as compared to control (**Table 1**). Two of these proteins, mitofilin ($-65\pm 2\%$), associated with mitochondrial cristae organization (John et al., 2005), and ubiquitous mitochondrial creatine kinase (MtCK) ($-82\pm 3\%$), associated with ADP-ATP exchange and the permeability transition pore (Beutner et al., 1998; Schlattner et al., 1998; Vyssokikh and Brdiczka, 2003), participate in mitochondrial structure maintenance. Both proteins exhibited a strong decrease in fluorescence intensity following exposure of mitochondria to DAQ (**Figure 2D-E**), with MtCK exhibiting the largest changes in relative fluorescence in Cys-CyDye 2-D

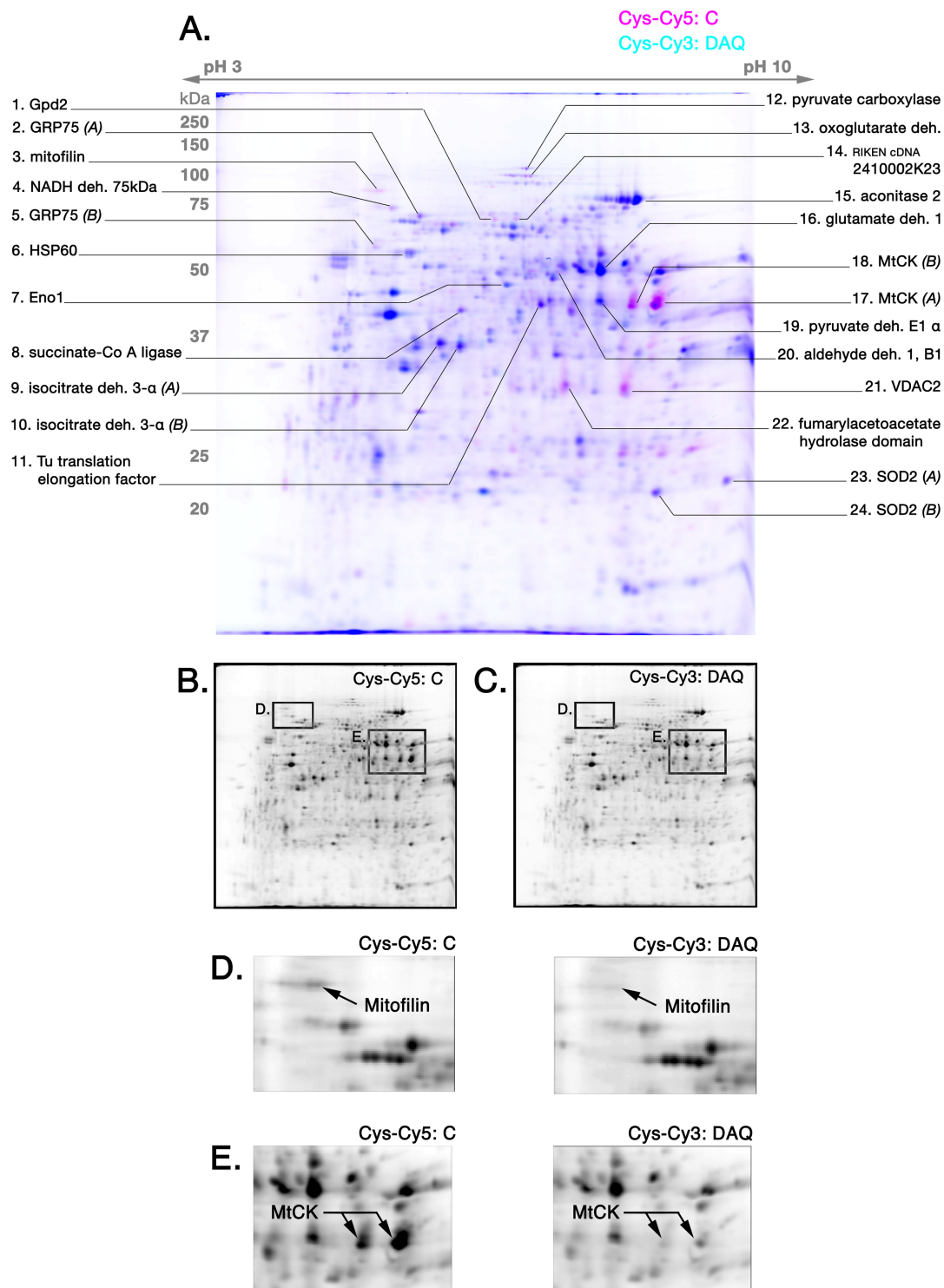


Figure 2: 2-D DIGE using Cys-CyDye labeling.

Figure 2 (continued): 2-D DIGE using Cys-CyDye labeling.

Isolated brain mitochondria were exposed to DA (150 μ M)/tyrosinase (150U) for 15 min. Lysed control (C) and DAQ-exposed (DAQ) mitochondrial proteins were reacted separately with a minimal concentration of Cys-Cy5 and Cys-Cy3 CyDyes, respectively, and then analyzed by 2-D DIGE. (A) A representative Cys-CyDye DIGE gel of control (**Cys-Cy5**, pseudocolored magenta) and DAQ-exposed (**Cys-Cy3**, pseudocolored cyan) mitochondrial protein. Spots exhibiting differential labeling present as magenta (decreased following DAQ) or cyan (increased following DAQ) hue, while blue spots represent minimal or no differential labeling. Specific protein spots of interest were picked from the gel and identified via MS analysis, and identities are presented with their associated spot (deh. – dehydrogenase). (B, C) Insets of separate black & white images obtained from ImageQuant of the DIGE gel for Cy5 (C) and Cy3 (DAQ-exposed) fluorophores. The pseudocolor overlay of B and C generated the image in A. (D) Magnified views of the region containing the identified protein spot mitofilin, comparing fluorescence intensity between Cys-Cy5 labeled (C) and Cys-Cy3 labeled (DAQ-exposed) images of the gel. (E) Magnified views of the region containing the identified protein spot mitochondrial creatine kinase (MtCK), comparing fluorescence intensity between Cys-Cy5 labeled (C) and Cys-Cy3 labeled (DAQ-exposed) images of the gel. These images demonstrate a decrease in Cys-CyDye fluorescent labeling following mitochondrial DAQ exposure for both mitofilin and MtCK.

DIGE gels. Other identified proteins whose relative fluorescence intensities were significantly reduced included several proteins of the Krebs cycle, including pyruvate carboxylase (-36 \pm 6%), succinate-CoA ligase (-43 \pm 4%), and oxoglutarate dehydrogenase (-49 \pm 5%), a protein previously shown to be inhibited by reactive metabolites of 5-cysteinyl-DA (Shen et al., 2000).

When DAQ exposure to mitochondria was carried out in the presence of 1mM N-acetylcysteine, an antioxidant, no differentially labeled spots were observed with Cys-CyDye DIGE (data not shown), demonstrating that DA oxidation is necessary to elicit the differential labeling of proteins. However, Cys-CyDye DIGE analysis alone cannot distinguish whether the

reductions in labeling intensity of a specific protein are the result of a change in the redox state of its thiols by oxidation/modification by DAQ, or a change in protein abundance. We utilized DIGE-compatible lysine-reactive fluorescent dyes to probe this distinction.

3.4.2 Lysine-reactive CyDye DIGE Reveals Differential Fluorescent Labeling Comparable to Cysteine-reactive CyDye DIGE

As with the Cys-CyDye DIGE experiments, Lys-CyDye DIGE analysis revealed a reproducible pattern of protein spots (**Figure 2A**; n=6), within which a subset of observed protein spots displayed differential fluorescence intensity. Further, Lys-CyDye labeled DIGE gels resulted in an overall protein spot pattern similar to Cys-CyDye labeled DIGE gels (**Figures 2 & 3**), as confirmed by MS analysis and peptide mass fingerprinting of spots from Lys-CyDye gels. In addition, proteins of interest demonstrated a similar differential fluorescence with both Cys- and Lys-CyDye 2-D DIGE (**Figure 3**). The proteins mitofilin and MtCK, in particular, both demonstrated a noticeable decrease in Lys-CyDye fluorescence intensity following DAQ exposure as compared to control (**Figure 3D-E**).

Quantitative DeCyder analysis across a set of Lys-CyDye gels (n=7) confirmed the significantly decreased fluorescence labeling of the subset of differentially labeled proteins in DAQ-exposed mitochondria as compared to control (**Table 1**). DeCyder analyses also demonstrate that the values of percent of control are comparable for all identified differentially labeled proteins between Cys- and Lys-CyDye 2-D DIGE (**Figure 4**). In particular, the proteins MtCK, mitofilin, fumarylacetoacetate hydrolase domain containing 2A, voltage-dependent anion

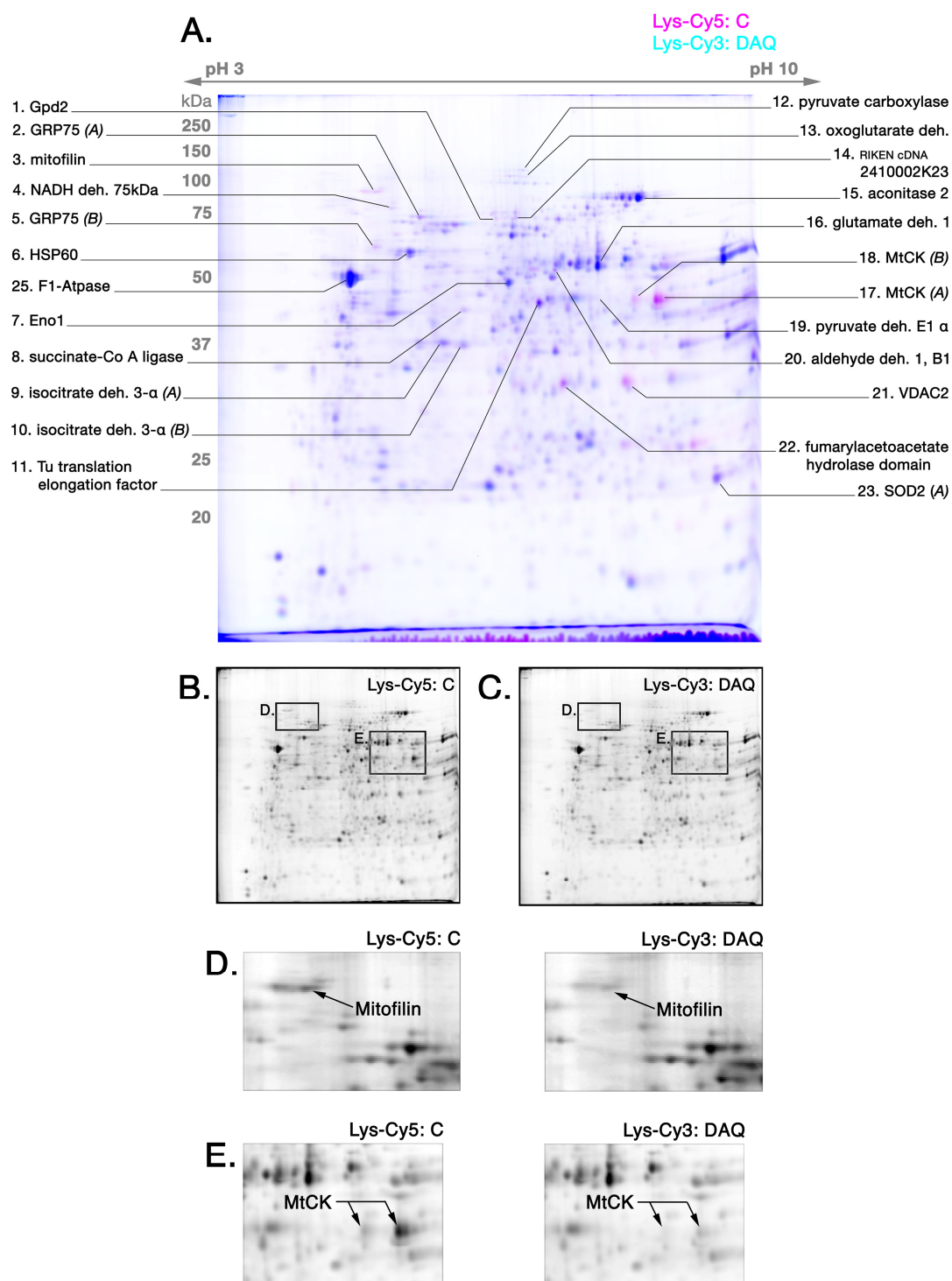


Figure 3: 2-D DIGE using Lys-CyDye labeling.

Figure 3 (continued): 2-D DIGE using Lys-CyDye labeling.

Isolated brain mitochondria were exposed to DA (150 μ M)/tyrosinase (150U) for 15 min, lysed control (C) and DAQ-exposed (DAQ) mitochondrial proteins reacted separately with a minimal concentration of Lys-Cy5 and Lys-Cy3 CyDyes, respectively, and then analyzed by 2-D DIGE. (A) A representative Lys-CyDye DIGE gel of control (**Lys-Cy5**, pseudocolored magenta) and DAQ-exposed (**Lys-Cy3**, pseudocolored cyan) mitochondrial protein. Spots exhibiting a magenta or cyan hue represent differential labeling indicative of alterations in protein level, while blue spots represent minimal or no differential labeling. Specific protein spots of interest were picked from the gel and identified via MS analysis, and confirmed identities are presented with their associated spot (deh. – dehydrogenase). (B, C) Insets of separate black & white images obtained from ImageQuant of the DIGE gel for Cy5 (C) and Cy3 (DAQ-exposed) fluorophores. The pseudocolor overlay of B and C generated the image in A. (D) Magnified views of the region containing the identified protein spot mitofilin, comparing fluorescence intensity between Lys-Cy5 labeled (C) and Lys-Cy3 labeled (DAQ-exposed) images of the gel. (E) Magnified views of the region containing the identified protein spot mitochondrial creatine kinase (MtCK), comparing fluorescence intensity between Lys-Cy5 labeled (C) and Lys-Cy3 labeled (DAQ-exposed) images of the gel. These images demonstrate a decrease in Lys-CyDye fluorescent labeling following mitochondrial DAQ exposure for both mitofilin and MtCK.

channel 2 (VDAC2), and glycerol-3-phosphate dehydrogenase all exhibit fluorescence labeling decreased greater than 50% in both Cys- and Lys-CyDye DIGE analyses, and represent the proteins with the greatest decrease in fluorescence intensity in mitochondria exposed to DAQ as compared to control. These findings suggest that alterations demonstrated by our 2-D DIGE analyses result primarily from decreases in a specific subset of proteins, and not from oxidation of thiols.

Table 1. Protein alterations in isolated rat brain mitochondria exposed to DAQ

Protein Spot	Protein	Protein Identification	NCBI Accession #	Predicted Protein MW; pI	Protein Score; C.I.%**	Peptide Count	% Coverage	Cys-CyDye (% of control, \pm SEM)#	Lys-CyDye (% of control, \pm SEM)#
1	Gpd2	Gpd2 protein [Rattus norvegicus]	gi 54035427	80921.3; 6.18	101; 100%	17	31%	32.3 \pm 2.74*	46.6 \pm 4.55*
2	GRP75 (A)	grp75 [Rattus sp.]	gi 1000439	73983.9; 5.87	243; 100%	27	53%	78.8 \pm 2.60*	73.7 \pm 3.30*
3	mitofilin	similar to inner membrane protein, mitochondrial [Rattus norvegicus]	gi 34855983	82304.9; 5.37	199; 100%	26	42%	35.2 \pm 2.39*	45.8 \pm 2.51*
4	NADH deh. 75kDa	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75 kDa [Rattus norvegicus]	gi 51858651	79361.6; 5.65	245; 100%	30	49%	54.5 \pm 3.24*	54.1 \pm 3.48*
5	GRP75 (B)	dnaK-type molecular chaperone grp75 precursor - rat	gi 2119726	73698.8; 5.87	141; 100%	19	42%	73.3 \pm 3.41*	75.1 \pm 3.35*
8	succinate-CoA ligase	PREDICTED: similar to succinate-Coenzyme A ligase, ADP-forming, beta subunit [Rattus norvegicus]	gi 62661722	50274.1; 7.57	156; 100%	20	52%	57.0 \pm 4.48*	57.9 \pm 2.22*
9	isocitrate deh. 3-alpha (A)	isocitrate dehydrogenase 3 (NAD+) alpha [Rattus norvegicus]	gi 16758446	39588; 6.47	89; 99.996%	13	37%	86.1 \pm 2.28	80.6 \pm 1.95*
11	Tu translation elongation factor	PREDICTED: similar to Tu translation elongation factor, mitochondrial [Rattus norvegicus]	gi 109462848	49890.1; 7.23	209; 100%	21	65%	72.7 \pm 2.26*	59.1 \pm 1.43*
12	pyruvate carboxylase	Pc protein [Rattus norvegicus]	gi 55716041	129694.7; 6.34	213; 100%	31	39%	63.9 \pm 5.90*	69.0 \pm 1.71*
13	oxoglutarate dehydrogenase	Similar to oxoglutarate dehydrogenase (lipoamide) [Rattus norvegicus]	gi 53734284	116221.4; 6.3	85; 99.99%	18	21%	50.5 \pm 4.91*	54.8 \pm 3.77*
14	RIKEN cDNA 2410002K23	similar to RIKEN cDNA 2410002K23 [Rattus norvegicus]	gi 34868689	80410.8; 6.56	114; 100%	19	29%	74.0 \pm 7.00*	56.1 \pm 5.17*
17	MtCK (A)	ubiquitous mitochondrial creatine kinase [Rattus rattus]	gi 57539	46999.3; 8.72	115; 100%	16	49%	18.1 \pm 2.71*	24.6 \pm 2.11*
18	MtCK (B)	creatine kinase, mitochondrial 1, ubiquitous [Rattus norvegicus]	gi 60678254	46932.2; 8.58	128; 100%	17	54%	26.6 \pm 3.24*	41.4 \pm 2.93*
20	aldehyde deh. 1, B1	Aldehyde dehydrogenase 1 family, member B1(predicted) [Rattus norvegicus]	gi 51858643	58101.6; 6.62	125; 100%	15	43%	N.D.***	64.2 \pm 3.94*
21	VDAC2	B-36 VDAC 36 kda voltage dependent anion channel [rats, hippocampus, Peptide, 295 aa]	gi 299036	31699.6; 7.44	56; 94.448%	8	40%	25.5 \pm 3.56*	31.7 \pm 3.25*
22	fumarylacetoacetate hydrolase domain	PREDICTED: similar to fumarylacetoacetate hydrolase domain containing 2A [Rattus norvegicus]	gi 34858672	40314; 8.49	122; 100%	15	56%	34.9 \pm 2.61*	43.9 \pm 2.09*
23	SOD2 (A)	unnamed protein product [Rattus norvegicus] (Superoxide dismutase 2)	gi 56691	24667.6; 8.96	126; 100%	13	65%	65.2 \pm 3.06*	64.6 \pm 2.35*
24	SOD2 (B)	unnamed protein product [Rattus norvegicus] (Superoxide dismutase 2)	gi 56691	24667.6; 8.96	59; 96.016%	8	41%	72.3 \pm 2.67*	N.D.****

* Significance from control (100%), p<0.01, for proteins outside of the cutoff of 83.3-120% of control (1.2 fold change)

** Probability-based MOWSE score (Protein Score) and Protein Score Confidence Interval (C.I.) represent the top Protein Score and C.I. pairing obtained across

all gels, Cys- and Lys-CyDye, in

which the protein was confidently identified (n = 2-11)

*** Insufficient data for DeCyder analysis

**** Protein not identified via MS analysis in Lys-DIGE gels

Normalized fold change in fluorescence of DAQ sample compared to control as determined by DeCyder analysis, expressed as percent of control (100%) \pm standard error of the mean (SEM)

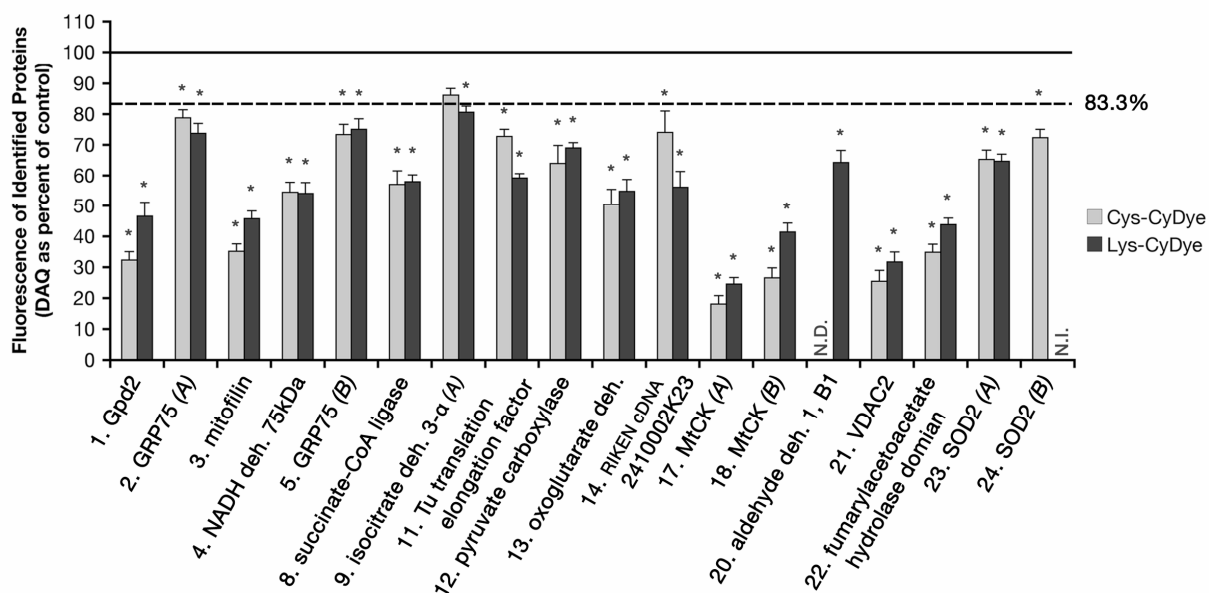


Figure 4: Quantitative analyses of Cys-CyDye and Lys-CyDye fluorescence intensities of mitochondrial proteins following DAQ.

Cy5 and Cy3 images obtained from the Typhoon 9400 scanner for DIGE gels were analyzed by comparison using DeCyder software. Normalized values of fold change in fluorescence obtained from DeCyder were converted to fluorescence of proteins following DAQ as percent of control. The graph shows the mean percent of control value for annotated protein spots in the Cys-CyDye gel in Figure 1 (Cys-CyDye) and the Lys-CyDye gel in Figure 2 (Lys-CyDye). N.D. indicates insufficient data for analysis. N.I. indicates that MS data was insufficient to verify identification of SOD2 (B) in Lys-CyDye DIGE gels. Proteins with differential fluorescence below a cutoff of 83.3% of control (1.2 fold change) were considered changed and analyzed for significance from control. (mean \pm SEM; n= 6 Cys-CyDye DIGE; n= 7 Lys-CyDye DIGE; *, significance from control (100%), $p < 0.01$)

3.4.3 Comparison of Lys- and Cys-Dye DIGE

Reciprocal labeling experiments with the Cys-CyDyes (n=3) and Lys-CyDyes (n=5) confirmed that the differential labeling was not influenced by preferential binding by the dye (data not shown). Most of the subset of proteins showing altered levels of labeling has been successfully identified (**Table 1**), though due to limitations in detection sensitivity, we were unable to identify all proteins exhibiting differential labeling. Despite this, it was obvious that the vast majority of mitochondrial proteins do not show any change in labeling following DAQ exposure (**Figures 2A and 3A**). As only a fraction of the total observable spots on the 2-D DIGE gel exhibited a significant decrease in fluorescence, these proteins represent a unique subset of the mitochondrial proteome that are susceptible to alterations induced by DAQ exposure and result in decreased protein levels.

These data suggest the effect of DAQ exposure quickly results in reduced levels of specific proteins, but the exact mode of DAQ-induced alterations of these proteins is unclear. In an attempt to block possible proteolytic degradation, mitochondria were exposed to DAQ in the presence of a 12.5-fold greater concentration of protease inhibitor cocktail than normally used, but this did not have any apparent effect on the observed changes in protein levels as detected with Cys- or Lys-CyDyes (data not shown).

To our knowledge, this is the first study to examine the use of Cys-CyDyes in a minimal-labeling scheme as compared to the more-typically utilized Lys-CyDyes. The maleimide moiety has a high reactivity with and selectivity for cysteine residues (and lesser reactivity with primary amines), and Cys-CyDyes are typically used under saturation-labeling conditions (Shaw et al., 2003). Although we initially anticipated that we would observe differential labeling indicative of oxidative modification of cysteinyl residues, minimal labeling using Cys-CyDyes detected only

alterations in protein levels nearly identical to labeling by Lys-CyDyes. Thus, differential labeling observed in Cys- and Lys-CyDye DIGE gels represents changes in abundance of proteins.

The possibility exists that while cysteines are likely being modified by exposure to DAQ, DAQ-induced modification may be below detection limits under our experimental conditions. Data from a parallel 2-D DIGE study of mitochondria isolated from PC12 cells exposed to DA similarly indicate that using Cys-CyDyes at low concentrations primarily detects changes in protein levels (Dukes et al., 2008). Although a reasonable approach in theory, labeling and detection of bulk protein thiols as conducted here and investigated by others (Chan et al., 2005; Hurd et al., 2007) is not an effective method for detecting changes in thiol redox state.

It is important to note that under manufacturer-recommended protocols, purchased quantities of both Lys-CyDyes and Cys-CyDyes are only sufficient for five to ten experiments. The Cys-CyDye minimal labeling methodology utilizes only a fraction of the recommended dye concentration, and increases the number of possible experiments that can be performed for a single purchased set of dyes by as much as 800 fold. This methodology, however, does have the drawback that it can only detect proteins containing reactive cysteine residues. Thus controls, such as Lys-CyDye DIGE or Western blot analyses, would be necessary to validate any observations or to evaluate proteins lacking cysteine residues. Nevertheless, with increased cost effectiveness, the methodology described here may make the Cys-CyDyes an ideal choice for certain DIGE experiments.

3.4.4 Comparable Decreases in Mitofilin and MtCK Levels Observed by Western Blot

To confirm the decreases in protein levels observed in the DIGE experiments, Western blot analyses of two representative proteins, mitofilin and MtCK, were carried out on mitochondrial samples treated with the same exposure to DAQ as the DIGE experiments. Comparing lysates from control and DAQ-exposed rat brain mitochondria on Western blot, we observed significant decreases of both mitofilin and MtCK protein levels ($-63.4 \pm 5.2\%$, $n=7$, and $-51.4 \pm 7.5\%$, $n=6$, respectively, $P < 0.05$) comparable to DIGE observations following exposure to DAQ (**Figure 5A-B**). We also observed immunoreactive bands at higher molecular weights for both MtCK and mitofilin after exposure to DAQ (**Figure 5C-D**), suggesting possible protein aggregation or crosslinking.

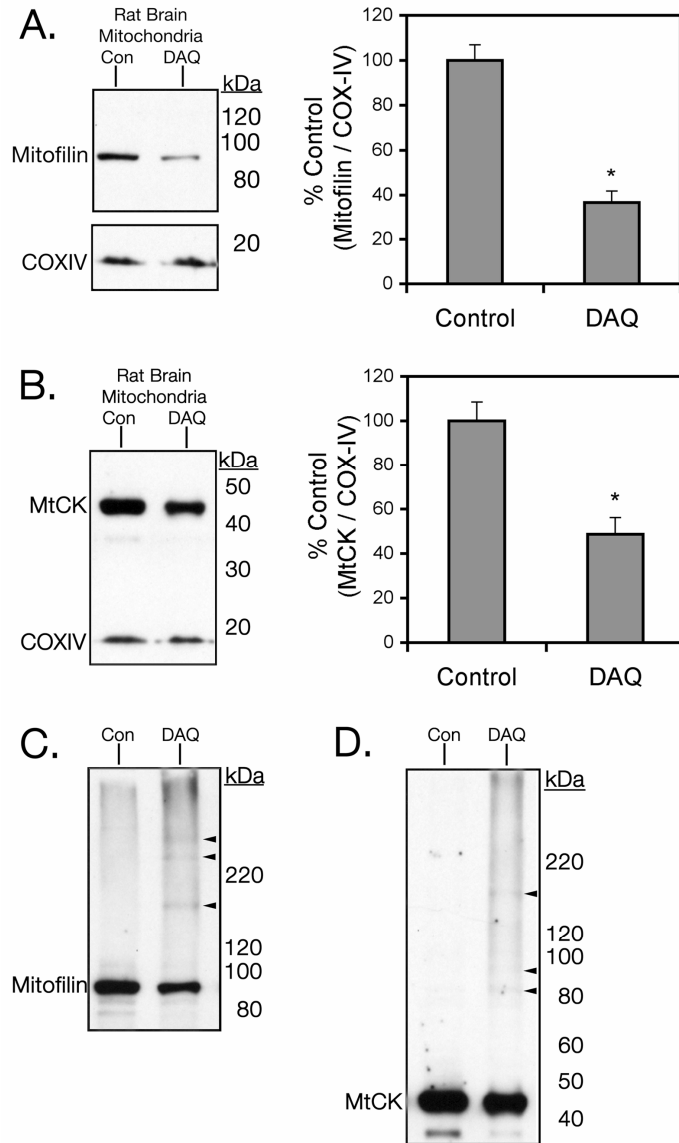


Figure 5: Confirmation of protein level alteration following mitochondrial DAQ exposure.

Western blot analysis was utilized to confirm DIGE analysis results for mitochondrial creatine kinase (MtCK) and mitofilin. Isolated mitochondria were exposed to DAQ, then lysed and subjected to SDS-PAGE and Western blot analysis. Representative Western blots of (A) mitofilin (n=7) and (B) MtCK (n=6) from intact rat brain mitochondria, Control (Con) versus DAQ (DAQ), are presented with densitometry analysis representing DAQ-exposed band density as percent of Control band density. Data is represented as a ratio of detection of the loading control, COXIV (mean \pm SEM; *, significant from control, $p < 0.05$). Extended film exposure revealed higher molecular weight detection (arrowheads) of (C) mitofilin and (D) MtCK in isolated mitochondria following DAQ.

3.4.5 Decreased Mitochondrial Levels of Mitofilin and MtCK in DA-Exposed PC12 Cells

To examine whether the DA-induced decrease in mitofilin and MtCK translates to the cellular environment, we utilized an established model of DA-induced PC12 cell toxicity. Mitochondria were isolated from differentiated PC12 cells following exposure to control media or media containing 150 μ M DA for 16 hr. This concentration and time point were previously demonstrated in our laboratory to result in covalent modification of cellular proteins by DAQ (indicative of DA oxidation) prior to significant cell death at 24 hrs (unpublished data). Lysates of mitochondria isolated from both treatment groups were subjected to SDS-PAGE and Western blot analyses. Data showed a significant decrease in both mitofilin and MtCK protein levels ($-15.5 \pm 5.8\%$ and $-25.7 \pm 5.4\%$, respectively, $n=4$, $P<0.05$) in mitochondria of DA-exposed PC12 cells as compared to controls (**Figure 6A-B**). Also, as observed in isolated rat brain mitochondria exposed to DAQ, higher molecular weight bands were detected for mitofilin in mitochondria from DA-exposed PC12 cells (**Figure 6C**), indicative of potential protein crosslinking and/or aggregation.

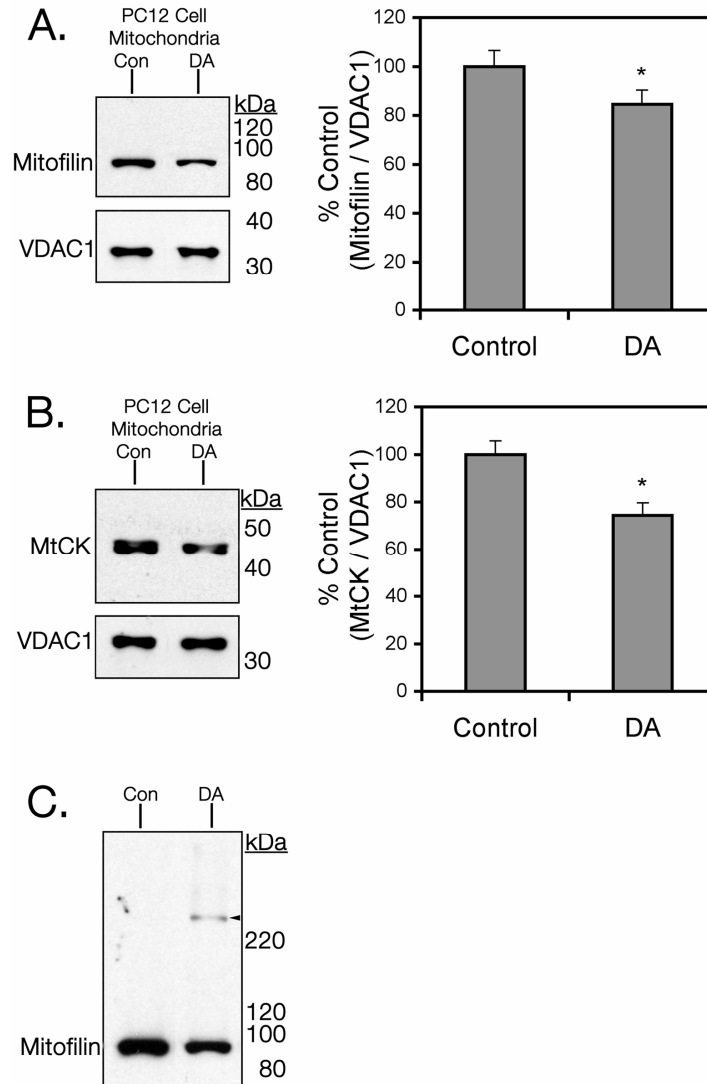


Figure 6: Detection of mitofilin and MtCK in PC12 cell mitochondria following DA exposure.

Control and DA-exposed PC12 cell mitochondria were collected, lysed, and subjected to SDS-PAGE and Western blot analysis using VDAC1 as a protein loading control. Representative Western blots of (A) mitofilin and (B) MtCK in mitochondria from PC12 cells, Control (Con) versus DA are presented with densitometry analysis representing band density of DA-exposed as percent of Control band density. Data is represented as a ratio of detection of the loading control, VDAC1 (mean \pm SEM, n=4; *, significant from control, $p < 0.05$). (C) Extended film exposure revealed higher molecular weight detection (arrowhead) of mitofilin in PC12 cell mitochondria following DA.

3.5 DISCUSSION

3.5.1 Summary

Oxidation of DA to reactive metabolites, ROS, and DAQ is thought to contribute to the oxidative stress, mitochondrial dysfunction, and dopaminergic neuron degeneration in PD. In this study, we sought to identify mitochondrial proteins susceptible to DA oxidation using an unbiased proteomics approach. We found that exposure of isolated brain mitochondria to reactive DAQ resulted in a rapid loss of specific proteins. The altered proteins identified in this study (listed in **Table 1**) encompass a range of mitochondrial functions including structural maintenance, transport, and metabolism, suggesting that DA oxidation may have detrimental effects on mitochondrial function. Indeed, loss or altered function of many of the proteins we have identified in this study have been previously associated with oxidative stress, mitochondrial dysfunction, and neurodegenerative diseases including PD (Beutner et al., 1998; Jin et al., 2006a; Kim et al., 2001; Myung et al., 2003; Scott, 2006; Suh et al., 2004; Vyssokikh and Brdiczka, 2003).

Two of the proteins whose abundance are most decreased following DAQ exposure as determined by 2-D DIGE analysis are MtCK and mitofilin, and their respective decreases in abundance were confirmed by Western blot analyses. Levels of both proteins were also significantly decreased in the mitochondria of PC12 cells exposed to DA, suggesting susceptibility of MtCK and mitofilin to DA oxidation. Western blot analyses also revealed the presence of higher molecular weight species immunoreactive for MtCK in rat brain mitochondria and for mitofilin in both rat brain and PC12 cell mitochondria following DA oxidation. These

results suggest increased protein aggregation and/or crosslinking as a potential consequence of oxidation by DAQ, though the nature of these interactions has yet to be examined.

3.5.2 Mitochondrial Creatine Kinase and Mitofilin Levels Are Altered Following Dopamine Oxidation

The protein whose relative fluorescence intensity was most decreased in our study, MtCK (-75% Lys-CyDye), is important in regulating ATP equilibrium in cells by generating phosphocreatine to help buffer against rapidly fluctuating energy usage (Schlattner et al., 1998). MtCK, an octameric protein, also plays a key role in mitochondrial morphology through formation and stabilization of contact sites between the inner and outer mitochondrial membranes (Lenz et al., 2007; Speer et al., 2005). The cysteine-dependent activity of MtCK is known to be highly sensitive to oxidative modification (Dolder et al., 2001). Oxidative stress may also result in dissociation of MtCK's octameric structure into dimers (Dolder et al., 2001; Wendt et al., 2003), disrupting contact sites and potentially facilitating opening of the mitochondrial permeability transition pore (PTP) (Brdiczka et al., 2006; Vyssokikh and Brdiczka, 2003). Thus, DA oxidation-induced modifications to MtCK activity or stability may have impacts on mitochondrial structural integrity and energy metabolism.

Another protein with significantly decreased abundance is the inner mitochondrial membrane protein mitofilin (-54% Lys-CyDye). The presence of mitofilin has been shown to be critical for maintenance of mitochondrial cristae structure (John et al., 2005), though the specific role of mitofilin in the mitochondria is unknown. A recent study suggested mitofilin forms a complex with mitochondrial proteins Sam50 and metaxins 1 and 2, integral in mitochondrial protein import (Xie et al., 2007), though the exact relationship mitofilin shares with these

proteins has not been evaluated. Various studies have shown that mitofilin is susceptible to oxidative stress, demonstrating oxidatively-modified cysteine and tryptophan residues (Suh et al., 2004; Taylor et al., 2003) and ROS-induced reduction of protein levels (Magi et al., 2004). A proteomic study on fetal Down syndrome brain tissue demonstrated a nearly 50% decrease in mitofilin, one of three proteins found to be significantly decreased in association with the disorder (Myung et al., 2003). Our study shows, using a non-biased approach, that mitofilin is susceptible to oxidative stress, resulting in decreased mitofilin protein levels. Such loss may impact mitochondrial structure and function, both of which are relevant to PD pathogenesis. Though the major function and interacting proteins of mitofilin are only beginning to be defined, a lot more remains to be understood about the important role of this protein within the mitochondria.

3.5.3 Proteins Associated With Mitochondrial Dysfunction and Neurodegeneration Are Altered Following Dopamine Quinone Exposure

The altered proteins we have identified cover a wide range of critical mitochondrial functions, and the inhibition of any one could be detrimental to mitochondrial function. Previous proteomic studies utilizing animal models of PD have also demonstrated alterations in mitochondrial proteins, including oxidation of key metabolism proteins (Poon et al., 2005b) and changes in expression and/or abundance of critical proteins (Jin et al., 2005; Palacino et al., 2004; Periquet et al., 2005). Several of the proteins described here, such as the Complex I 75 kDa subunit, VDAC2, and mortalin, have also displayed altered abundance in previous animal models of PD (Jin et al., 2005; Periquet et al., 2005).

The level of the NADH-ubiquinone oxidoreductase 75 kDa Fe-S protein, a key subunit of mitochondrial Complex I, was significantly decreased (-46% Lys-CyDye) in isolated rat brain mitochondria following exposure to DAQ. Mutation and deletion of the 75 kDa subunit are linked to mitochondrial encephalopathy (Benit et al., 2001; Bulgiani et al., 2004); and led to reduced levels of Complex I, reduced Complex I activity, and increased mitochondrial ROS accumulation in fibroblasts (Iuso et al., 2006). As previously discussed, Complex I dysfunction has been directly linked to PD, and inhibition of Complex I and the ETC are known to lead to further ROS production by the mitochondria (Beal, 2003). In the 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP)-treated mouse model of PD, proteomic analysis demonstrated a significant loss of the 75 kDa subunit in SN pars compacta tissue (Jin et al., 2005). Protein levels of the 75 kDa subunit were also significantly reduced in various brain regions of Down syndrome and Alzheimer's disease patients (Kim et al., 2001). Thus, loss of the 75 kDa subunit could be detrimental to cellular viability.

The voltage-dependent anion channel 2 (VDAC2) was greatly decreased (-68% Lys-CyDye) in fluorescence labeling following DAQ exposure in rat brain mitochondria. The VDAC proteins, like MtCK, have a role in the PTP. VDAC is also believed to be a key player in mitochondrial-mediated apoptosis (Shoshan-Barmatz et al., 2006). VDAC2, in particular, has been shown to regulate the proapoptotic molecule BAK (Cheng et al., 2003), a function that may be compromised if DA oxidation reduces protein levels. A previous study also observed a significant decrease of VDAC2 in the SN of MPTP-treated mice (Jin et al., 2005). While we found a decreased relative abundance of the VDAC2 isoform upon DAQ exposure, other isoforms of VDAC (VDAC1 and 3) were not detected. Thus, further analyses will be necessary to evaluate whether these isoforms are equally susceptible to DAQ-induced changes.

The mitochondrial protein chaperone, heat shock protein 70/glucose regulated protein75/mortalin (mortalin), another protein whose levels were demonstrably decreased (-26% Lys-CyDye) in this study, has also been linked to PD. Mortalin serves as an important chaperone in mitochondria, with a major role in mitochondrial protein import and folding (Geissler et al., 2001). A recent proteomic study found mortalin abundance to be decreased in SNpc of PD patients and in MES cells exposed to the mitochondrial Complex I inhibitor rotenone (Jin et al., 2006a). Mortalin was also shown to interact with PD-related proteins DJ-1 and α -synuclein in cultured cells (Jin et al., 2007), with enhanced DJ-1 interaction following oxidative stress (Li et al., 2005). These studies combined with our present findings suggest that mortalin protein interactions and abundance are susceptible to oxidative stress. Thus, it is possible that loss of this protein would have a major impact on importation and incorporation of key mitochondrial proteins, particularly at times of stress. The exact role of mortalin in PD pathogenesis, however, remains to be elucidated.

The cause of the loss of specific proteins identified in this study has yet to be determined. One source may be rapid aggregation of oxidatively modified proteins. The Western blot analyses of mitofilin and MtCK indicate that protein aggregation or covalent protein-protein interactions do occur rapidly following *in vitro* DAQ exposure (15 min). Another possibility may be the rapid proteolytic degradation of oxidatively modified proteins. It is well established that mitochondria contain proteases dedicated to degradation of misfolded, denatured, and oxidatively modified proteins (Bota and Davies, 2001; Bota and Davies, 2002). While preliminary experiments using increased levels of protease inhibitors in the DAQ-exposure reaction did not appear to affect protein loss, protection by protease inhibitors may be

confounded by the presence of intact mitochondrial membranes in our preparations. More work will be necessary to elucidate the nature of the observed protein loss.

3.5.4 Conclusions

Our results demonstrate that DA oxidation results in the loss of a select subset of mitochondrial proteins. Such an event, if not countered promptly, could lead to severely decreased mitochondrial function and stability. A major significance of the MtCK and mitofilin alterations observed may be in the roles of these proteins in maintaining mitochondrial morphology (John et al., 2005; Lenz et al., 2007; Speer et al., 2005). Dopamine oxidation-induced alterations in MtCK and mitofilin may lead to disruption of key protein-protein interactions and to mitochondrial structure reorganization.

Given the functions of proteins such as MtCK, mitofilin, and mortalin, and their responses to oxidative stress, it is possible that mitochondrial protein alterations resulting from DA oxidation may lead to impaired mitochondrial protein import, cristae reorganization, and PTP formation. The mitochondrial state may, in turn, deteriorate further due to alterations in key proteins involved in the Krebs cycle and ETC function, including the 75 kDa subunit of Complex I, leading to increased ROS formation. This could create a vicious cycle of oxidative damage, resulting in amplified mitochondrial dysfunction and, ultimately, neuronal degeneration and disease progression. Further study will be necessary to evaluate this hypothesis and the specific roles for the proteins identified in this study within mitochondrial dysfunction. Characterizing the susceptibility of mitochondrial proteins to DA oxidation may be key to understanding the contribution of DA to the progression of PD pathogenesis, and for developing novel therapeutic strategies for PD treatment.

3.6 ACKNOWLEDGEMENTS

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4.0 PROTEOMIC IDENTIFICATION OF DOPAMINE-CONJUGATED PROTEINS FROM ISOLATED RAT BRAIN MITOCHONDRIA AND SH-SY5Y CELLS

4.1 SUMMARY

Dopamine oxidation has been previously demonstrated to cause dysfunction in mitochondrial respiration and membrane permeability. Mitochondrial dysfunction may be related to the covalent modification of critical proteins by the reactive dopamine quinone, though specific mitochondrial protein targets have not been identified. In this study, we utilized proteomic techniques involving two-dimensional gel electrophoresis, fluorescent labeling, autoradiography, and mass spectrometry to identify proteins directly conjugated with ^{14}C -dopamine following exposure of isolated rat brain mitochondria and differentiated SH-SY5Y human neuroblastoma cells to radiolabeled dopamine quinone and dopamine, respectively. We observed a subset of rat brain mitochondrial proteins that were highly susceptible to covalent modification by ^{14}C -dopamine, including chaperonin (HSP60), ubiquinol-cytochrome c reductase core protein 1, glucose regulated protein 75/mitochondrial HSP70/mortalin, mitochondrial creatine kinase, and isocitrate dehydrogenase 3 alpha. We used Western blot detection to confirm the association of radiolabeled dopamine with two proteins, mitochondrial creatine kinase and mitofilin. We also found the Parkinson's disease associated proteins ubiquitin carboxy-terminal hydrolase L1 (UCH-L1) and DJ-1 to be covalently modified by

dopamine in our brain mitochondrial preparations and in SH-SY5Y cells. The susceptibility of the identified proteins to covalent modification by dopamine may carry implications for their role in the vulnerability of dopaminergic neurons in Parkinson's disease pathogenesis.

4.2 INTRODUCTION

Mitochondrial dysfunction and oxidative stress have been implicated in the pathogenesis of neurodegenerative diseases, including Parkinson's disease (PD) (Beal, 2007; Halliwell, 2001; Halliwell, 2006; Jenner, 2003; Schapira, 2008). PD is a progressive disorder, pathologically characterized by the loss of pigmented dopaminergic neurons in substantia nigra (SN), and the formation of proteinaceous cytoplasmic inclusions called Lewy bodies (Samii et al., 2004). Though other brain regions are known to be involved in PD, the degeneration of the nigrostriatal dopaminergic neurons combined with the increased oxidative stress observed in PD suggests the neurotransmitter dopamine (DA) may be contributing to disease progression (Greenamyre and Hastings, 2004; Ogawa et al., 2005; Stokes et al., 1999).

Normal DA metabolism can lead to the production of reactive oxygen species (ROS). If not adequately stored in vesicles, DA is also prone to auto- or enzymatic oxidation in the cellular environment, leading to the formation of reactive DA quinone (DAQ) and additional ROS (Graham et al., 1978; Hastings, 1995). The electron deficient DAQ is readily susceptible to attack by cellular nucleophiles, predominantly reduced sulfhydryls abundantly found in cells on reduced glutathione, free cysteine, and cysteinyl residues of proteins (Tse et al., 1976). The interaction of protein with reactive DA metabolites will result in either covalent binding of thiols by DAQ to form 5-cysteinyl-DA, or oxidation of protein sulfhydryl groups (Graham et al., 1978;

Hastings et al., 1996; Ito et al., 1988). Many vital cellular and mitochondrial proteins contain cysteine residues whose redox states are critical for function. Thus, DA-induced oxidative modifications, which may alter protein structure as well as function, could have detrimental effects on the cell (Berman and Hastings, 1999; LaVoie and Hastings, 1999; Premkumar and Simantov, 2002).

DA-induced toxicity has been demonstrated both *in vitro* in cell culture (Ben-Shachar et al., 2004; Jones et al., 2000; Koshimura et al., 2000; Lai and Yu, 1997a; Offen et al., 1996) and *in vivo* (Hastings et al., 1996; Rabinovic et al., 2000), where toxicity to DA terminals was correlated to the amount of DA oxidation and modification of proteins (Hastings et al., 1996). DA and DAQ exposures also altered mitochondrial respiration in isolated intact rat brain mitochondria (Berman and Hastings, 1999; Cohen et al., 1997; Gluck et al., 2002) and triggered permeability transition (Berman and Hastings, 1999), suggesting modification of critical mitochondrial proteins.

Several cellular and mitochondrial proteins have been reported to have altered function following DA exposure and DA oxidation, including cytosolic and mitochondrial creatine kinase (Maker et al., 1986; Miura et al., 1999), mitochondrial aldehyde dehydrogenase (Turan et al., 1989), mitochondrial Complex I (Ben-Shachar et al., 2004; Khan et al., 2005), tyrosine hydroxylase (Kuhn et al., 1999; Xu et al., 1998), and the dopamine transporter DAT (Berman et al., 1996). Proteins associated with familial PD, parkin (LaVoie et al., 2005) and alpha-synuclein (Conway et al., 2001), have also been demonstrated to be targets of covalent modification by DA. It has also been shown that isolated brain mitochondria can accumulate exogenous radiolabeled DA (Brenner-Lavie et al., 2008). In addition, Kahn et al. demonstrated that exposure of crude mitochondrial-synaptosomal fractions to DA led to protein crossing-

linking and protein-bound DA formation (Khan et al., 2001). To date, however, the specific proteins directly modified by DAQ have not been identified. Thus, it was of interest to us to identify and characterize the mitochondrial targets of DA oxidation.

Proteomic techniques allow for the analysis and identification of modified proteins within the complete proteome of tissues or organelles. In recent years, proteomics has become a valuable and diverse tool in investigating disease processes of neurodegeneration, including altered protein expression and modification (Butterfield et al., 2006; Dalle-Donne et al., 2005; Fountoulakis and Kossida, 2006; Johnson et al., 2005; Kim et al., 2004). Previous studies combining techniques such as two-dimensional (2-D) gel electrophoresis and Western blot analysis have detected oxidatively modified proteins associated with neurodegenerative diseases in animal models and human post mortem tissue (Castegna et al., 2002a; Choi et al., 2004a; Poon et al., 2005b). Such techniques can also be applied toward the identification of proteins covalently modified by DAQ.

Recently, we have shown that several mitochondrial proteins are decreased in abundance following *in vitro* DAQ exposure (Van Laar et al., 2008; see **Chapter 3**). While demonstrating the effect of DAQ on a subset of mitochondrial proteins, the method utilized did not identify proteins directly modified by covalent binding with DAQ. In this study, we exposed isolated rat brain mitochondria to radiolabeled DA (^{14}C -DA) quinone, and differentiated SH-SY5Y neuroblastoma cells to exogenous ^{14}C -DA. Employing techniques including 2-D gel electrophoresis, autoradiography, and 2-D difference in-gel electrophoresis (DIGE) fluorescent labeling technology combined with mass spectrometry (MS) analysis, we have identified proteins directly conjugated to ^{14}C -DA. These findings further elucidate the effects of DA oxidation on cellular protein alterations, and may have implications for PD pathogenesis.

4.3 EXPERIMENTAL PROCEDURES

Materials

3,4-Dihydroxyphenylethylamine, [8-¹⁴C] (¹⁴C-DA) was purchased from MP Biomedicals, Inc. (Irvine, CA). IEF DryStrips (7 cm 3-5.6pH, 4-7pH, and 6-11pH, and 18 cm 3-10pH), and CyDye™ DIGE Fluor Scarce Sample Labeling (Cysteine-reactive) and Minimal Labeling (Lysine-reactive) dye kits were purchased from GE Healthcare (Piscataway, NJ). Bradford Dye Reagent was purchased from BioRad (Hercules, CA). Promega Gold Mass Spectrometry Grade Modified Trypsin was purchased from Promega (Madison WI). Dopamine (DA), Protease inhibitor cocktail (cat#P2714), mushroom tyrosinase, retinoic acid, and most general chemicals for SDS-PAGE, buffers, and solutions were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. The MtCK and mitofilin polyclonal antibodies used in this study were generated for our laboratory by Genemed Synthesis, Inc. (San Antonio, TX). Dulbecco's modified Eagle medium (DMEM; Gibco) cell culture media, fetal bovine serum (FBS; HyClone), and 10,000 U/mL penicillin/10,000 µg/mL streptomycin (pen/strep; Gibco) were purchased from Invitrogen (Carlsbad, CA). All general solutions and buffers were prepared using purified water from a Milli-Q system (Millipore Corp., Bedford, MA). Solutions for in-gel and on-blot trypsin digest procedures were prepared using HPLC-grade water from Fisher Biotech (Pittsburgh, PA), and HPLC-grade acetonitrile and spectrophotometric-grade methanol from Sigma-Aldrich (St. Louis, MO).

Mitochondrial Isolation and ¹⁴C-Dopamine Exposure Reactions

All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and are in accordance with guidelines put forth by the

National Institutes of Health in the *Guide for the Care and Use of Laboratory Animals*. Mitochondria were isolated from adult male Sprague-Dawley (300-350g) rat brain tissue via differential centrifugation as previously described (Van Laar et al., 2008; see **Chapter 3**). Mitochondrial pellets were resuspended in isolation buffer and kept on ice. Mitochondrial protein content was determined for the total suspension via Bradford protein assay (Bradford, 1976). Prior to experimental use, respiration measurements of the isolated mitochondria were made to ensure mitochondrial health, as previously described (Berman and Hastings, 1999). A state 3/state 4 ratio above 6 was considered an indication of healthy, intact mitochondria.

Mitochondrial protein (4 $\mu\text{g}/\mu\text{L}$) was exposed to 150 μM DA or ^{14}C -DA (0.5-1 μCi ; 150 μM) and tyrosinase (300U/mL), to oxidize DA to DAQ, in reaction buffer (225 mM mannitol, 75 mM sucrose, 25 mM HEPES, and 1 mM EGTA, pH 7.4 with PIC) for 15 min at room temperature (RT). Mitochondria were then pelleted by centrifugation at 15,000 g for 15 min at 0°C. Control mitochondria underwent an identical procedure without DA present. Mitochondrial pellets were lysed by rigorous pipetting in denaturing 2-D DIGE lysis buffer (9 M urea, 2% w/v CHAPS, and 30 mM Tris-base, pH 8.5) in a ratio of 100 μL buffer to 1 mg protein. Insoluble material was pelleted by centrifugation (16,000 x g for 1-2 min at RT) and discarded. Protein concentrations in the lysed control and DAQ-exposed samples were determined by the Bradford method (Bradford, 1976).

SH-SY5Y Cell Culture and Dopamine Exposure

Proliferating SH-SY5Y cells were maintained in DMEM supplemented with 10% FBS and 1% pen/strep (SH media). For differentiation, cells were subcultured onto 6 cm or 6-well plates at 2×10^5 cells/mL. Culture media was exchanged for fresh SH media supplemented with

20 μ M retinoic acid (SH differentiation media) 48 hr after plating, and every 48 hr thereafter for a total of 5 days of differentiation. On day 5, culture media was exchanged for fresh SH differentiation media supplemented with 150 μ M 14 C-DA (1 μ Ci/mL media) for 16 hr. Immediately following treatment, cells were collected by 1 min exposure to 0.25% trypsin with 2.21mM EDTA in HBSS (Mediatech; Herndon, VA) followed by force pipetting, rinsing with PBS, and centrifugation. The resulting cell pellet was rinsed with PBS, centrifuged, then lysed in DIGE lysis buffer supplemented with Chaps Cell Extract Buffer (Cell Signaling Technology; Danvers, MA) and PMSF.

2-D Gel Electrophoresis

For 2-D gel electrophoresis utilizing mini-gel SDS-PAGE, 100-250 μ g total protein from 14 C-DA exposed mitochondrial or SH-SY5Y cell lysate was loaded via sample cup on rehydrated 7cm pH 3-5.6, pH 4-7, or pH 6-11 DryStrips and isoelectrically focused on a Multiphor II electrophoresis unit according to manufacture's instructions (GE Healthcare). Focused strips were equilibrated for 10 min at RT in an equilibration buffer (75 mM Tris-HCl pH 6.8, 6 M urea, 30% v/v glycerol, 1% w/v SDS) supplemented with 30 mM DTT, followed by 10 min at RT in equilibration buffer supplemented with 240 mM iodoacetamide. Equilibrated strips were then subjected to electrophoresis on 12% SDS-PAGE gels utilizing a Hoefer Mighty Small II apparatus. Precision Blue markers (BioRad) were used as molecular weight standards.

Transblotting and Autoradiography of 14 C Dopamine-modified Proteins

Following 14 C-DA mini 2-D electrophoresis, proteins were transferred to PVDF membrane via a BioRad Trans-Blot Semi-Dry Electrophoretic Transfer system. For

autoradiography, blots were air-dried and recognizable landmarks were marked on the edges of the blot using a green-ink phosphorescent imaging marker (Diversified Biotech; Boston, MA) for later reference between the blot and the resulting autoradiogram. Blots were then placed in Kodak autoradiogram transcreens with BioMax MS autoradiogram film (Kodak) in a film exposure cassette at -80°C for 3 days to 3 weeks to allow for complete exposure.

Radiolabeled Protein Spot Excision and Trypsin Digest

Following autoradiography, PVDF blots were aligned with the developed film by matching the phosphorescent ink landmarks, and together they were placed on a transillumination light box. Using a sterile 2 mm tissue punch, points that corresponded with spots on the autoradiogram were excised from the blot. Excised PVDF spots were then subjected to trypsin digest by methods adapted from Bienvenut et al. (Bienvenut et al., 1999). Briefly, spots were washed with 1:1 methanol and water, and then air-dried. Membrane spots were then submerged in 10 μ L of 30% acetonitrile in 50 mM ammonium bicarbonate and 4 μ L of 0.1 mg/mL trypsin (Promega) and incubated 16-18 hr at RT with constant agitation. Following digestion, the supernatant was collected and saved in a separate tube. The membrane spots were then submerged in 20 μ L of 80% acetonitrile, and sonicated for 15 min in a sonicating waterbath at RT. The resulting supernatant was collected and added to the original digestion supernatant. The total supernatant was dried down by speed-vacuum without heating, and kept at 4°C under desiccation until MS analysis.

Cys- and Lys-CyDye labeling and 2-D DIGE

2-D DIGE analysis with either cysteine-reactive maleimide CyDye (Cys-CyDye) or lysine-reactive NHS-ester CyDye (Lys-CyDye) labeling of control and DAQ- or ^{14}C -DAQ-exposed mitochondrial protein was carried out as previously described (Van Laar et al., 2008; see **Chapter 3**). Briefly, controls were paired with DAQ or ^{14}C -DAQ reacted samples from the same mitochondrial isolation, and experiments were completed for sample sets from multiple mitochondrial isolations. Cy5-labeled control and Cy3-labeled DAQ- or ^{14}C -DAQ-exposed samples were combined in equal protein amounts, generating the following DIGE gel experiments: (1) Cys CyDye Cy5 control vs. Cy3 DA, (2) Cys CyDye Cy5 control vs. Cy3 ^{14}C -DA, (3) Lys CyDye Cy5 control vs. Cy3 DA, and (4) Lys CyDye Cy5 control vs. Cy3 ^{14}C -DA. We previously determined through reciprocal labeling that there is no disparate or preferential labeling exhibited by the individual dyes (Van Laar et al., 2008; see **Chapter 3**). Samples were isoelectrically focused on 18cm pH 3-10 DryStrips using a Multiphor II electrophoresis system (GE Healthcare). Focused DryStrips were equilibrated as described above and proteins were separated on 12% SDS-PAGE 1.5mm thick gels using a Hoefer SE600 Ruby Electrophoresis Unit.

Fluorescence Detection, Spot Picking, and In-gel Trypsin Digest

Immediately following the second dimension run, 2-D DIGE gels were scanned for Cy3 and Cy5 dye labeling using a Typhoon 9400 scanner with ImageQuant software (GE Healthcare) to obtain a 100-200 μm resolution image of the gel. Immediately following imaging, ^{14}C -DA 2-D DIGE gels were transblotted to PVDF or nitrocellulose. Non-radioactive 2-D DIGE gels were fixed overnight in 40% MeOH, 1% acetic acid solution at 4°C. Spots of interest were excised

using an automated spot picker, designed by Dr. Jonathan Minden of Carnegie Mellon University (instrumentation housed in the University of Pittsburgh Genomics and Proteomics Core Laboratories). Immediately following excision from 2-D DIGE gels, gel plugs were washed and carried through a previously described in-gel trypsin digest procedure (Van Laar et al., 2008; see **Chapter 3**).

Image Analysis

In-gel quantitative comparisons of fluorescence were completed utilizing DeCyder Difference In-Gel Analysis (DIA) module software (GE Healthcare). Fold change ratios, based on volume ratios of the individual spots and internally normalized by DeCyder, were determined and recorded for DeCyder-defined spots as previously described (Van Laar et al., 2008; see **Chapter 3**).

For ^{14}C -DA 2-D DIGE experiments, transblots were subjected to autoradiography. Digital scan images of the autoradiogram and the corresponding DIGE gel were digitally merged and compared visually. Images were aligned based on recognizable landmarks. Fluorescence-labeled protein spots that aligned with radioactive spots were considered spots of interest. For protein spots that we did not already have an identification for based on previous data (Van Laar et al., 2008; see **Chapter 3**), correlating spots were located on the non-radioactive DIGE gels and picked for identification, as described above.

MS and MS/MS Analyses for Protein Identification

For MS and MS/MS analyses, dried trypsin-digested samples were rehydrated in 2-3 μl of 0.3% trifluoroacetic acid, 1 mM ammonium citrate in 50:50 acetonitrile/water, and an equal

volume of saturated α -cyano-4-hydroxycinnamic acid matrix solution, and then spotted onto a MALDI target plate at 42°C. MS and MS/MS spectra were obtained using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA) and processed by GPS Explorer™ (ver. 3) data analysis software (Applied Biosystems) coupled with Mascot™ search engine (Matrix Science) for peak list generation, database search, and statistical analysis. MS and/or combined MS and MS/MS spectra were searched against the National Center for Biotechnology Information non-redundant (NCBI nr) and SwissProt databases, specifying “All Entries,” “*Rodentia*,” or “*Rattus*” species for rat brain mitochondria samples, and “All Entries” or “*Homo sapiens*” species for SH-SY5Y samples. In specifications for searches, trypsin digest was specified, and MS and MS/MS peak filtering were set at a minimum S/N ratio of 10, with a peak density filter of 50 and maximum 65 peaks allowed. Allowed mass ranges were 800-4000 Da for MS peaks and 20-60 Da under precursor for MS/MS. Precursor tolerance was set at 50 to 100 ppm and MS/MS fragment tolerance was set at 0.2 to 0.4 Da, allowing 1 missed cleavage. Modifications specified included fixed or variable cysteine carbamidomethylation and methionine oxidation.

For peptide mass fingerprinting via MS analysis, a positive protein identification was accepted when a top ranked hit yielded (1) a probability-based molecular weight search (MOWSE) protein score confidence interval percentage (C.I.%) > 95%, (2) peptides matched \geq 6, (3) a predicted molecular weight that was relative to the protein spot position on the gel or blot, and when (4) MS identification for the given ^{14}C -DA-labeled spot could be replicated across two or more separate experiments. Five statistically significant protein identities are also noted that were obtained via MS peptide mass fingerprinting analysis in only one ^{14}C -DA exposure experiment, though corresponding ^{14}C -DA conjugated spots were visible in blots from

multiple experiments. These identities are noted in **Tables 2** and **4**. Identification for a particular spot was accepted for combined MS and MS/MS (MS + MS/MS) results that yielded (1) a top ranked hit with both MS probability-based MOWSE protein score and MS/MS spectra total ion score C.I.% each > 95%, (2) total peptides matched ≥ 6 and/or significant ion score for ≥ 2 unique peptides, (3) a predicted molecular weight that was relative to the protein spot position on the blot, and (4) a corresponding ^{14}C -DA-labeled spot was present in blots from multiple experiments. Total ion scores were calculated from weighted ion scores for individual peptides that were matched to a given spot identity. C.I.% values are derived from the probability-based MOWSE scores. Thus, values >95% suggest identities are significant, and not random matches. For each protein identity provided, we ensured that the next non-homologous protein hit obtained from Mascot was non-significant, suggesting that only the protein identity provided occupied the spot picked (data not shown).

Western Blot Detection of Mitofilin & MtCK

Following autoradiography, the transblots generated from ^{14}C -DA 2-D DIGE gels were carried through the Western blot detection procedure. The membrane was washed and placed in a 1:1000 dilution of rabbit anti-MtCK or 1:5000 dilution of rabbit anti-mitofilin primary antibody 16-18 hr at 4°C. Membranes were developed using the BioRad Immune-Star® goat-anti-rabbit Chemiluminescence Detection kit, exposed to Biomax MR film (Kodak), and developed for imaging. Using recognizable landmarks for alignment, scanned images of the Western blot and the autoradiogram were digitally merged with the ImageQuant scan of the fluorescent DIGE gel for visual comparison.

4.4 RESULTS

4.4.1 ^{14}C -DA-conjugated mitochondrial proteins were identified directly from transblots of 2-D gels

Following exposure to ^{14}C -DAQ, mitochondria were lysed and proteins separated by 2-D gel electrophoresis using three separate pI ranges for the first dimension, pH 3-5.6 ($n = 5$ blots, from $n = 4$ mitochondrial experiments), pH 4-7 ($n = 9$ blots, from $n = 6$ mitochondrial experiments), and pH 6-11 ($n = 7$ blots, from $n = 6$ mitochondrial experiments). 2-D gels were transblotted to PVDF, and the membranes used to generate autoradiograms (**Figure 7**). Distinct spots of radioactivity indicated protein targets covalently modified by ^{14}C -DAQ. The autoradiograms were then aligned with their PVDF blots, corresponding ^{14}C -DA-associated spots were excised from the blot, and proteins were identified by mass spectrometry (MS) analysis (**Figure 7, Table 2**).

Proteins involved in various mitochondrial functions were identified as being covalently modified by ^{14}C -DAQ, including the tricarboxylic acid (TCA) cycle protein subunit isocitrate dehydrogenase 3-alpha, TCA cycle associated protein glutamate oxaloacetate transaminase 2, the NADH-ubiquinone oxidoreductase 30 kDa subunit of Complex I, the ubiquinol-cytochrome c reductase core protein 1 and Rieske Fe-S protein subunits of Complex III, ubiquitous mitochondrial creatine kinase (MtCK), and the chaperone proteins chaperonin, or heat shock protein 60 (HSP60), and mortalin, also known as glucose regulated protein 75 or mitochondrial heat shock protein 70 (mortalin/GRP75/mtHSP70). Additionally, three proteins previously demonstrated to interact with mitochondrial membranes, the glycolytic enzyme enolase (Brandina et al., 2006; Entelis et al., 2006; Giege et al., 2003) and the cytoskeletal proteins

tubulin (Carre et al., 2002) and actin (Boldogh and Pon, 2006; Boldogh and Pon, 2007), were also identified to be covalently modified following exposure to ^{14}C -DAQ. Note that three protein identities including mitochondrial voltage-dependent anion channel 1 (VDAC1), mitochondrial manganese superoxide dismutase (SOD2), and guanine nucleotide-binding protein subunit beta 2, a G-protein subunit that to our knowledge has not previously been associated with mitochondria, were confirmed by MS peptide mass fingerprinting analysis in only one blot each, though corresponding radiolabeled spots were present in blots from multiple experiments. Several other proteins not specifically mitochondrial were also found to be covalently modified by ^{14}C -DA, including the glycolytic enzyme triosephosphate isomerase, cytosolic creatine kinase, and the PD-associated proteins DJ-1 and ubiquitin carboxy-terminal hydrolase L1 (UCH-L1). The significance of the presence and modification of these proteins is discussed below.

The proteins tubulin, HSP60, and gamma-enolase were found in both pH 3-5.6 (**Figure 7A**) and pH 4-7 (**Figure 7B**) blots (likely due to overlap of the pI ranges in the first dimension separation), dually confirming the identity of these proteins as targets of covalent modification by ^{14}C -DAQ. The identity of some ^{14}C -DA-associated spots, however, remained unidentified due to limitations with protein recovery and MS analysis.

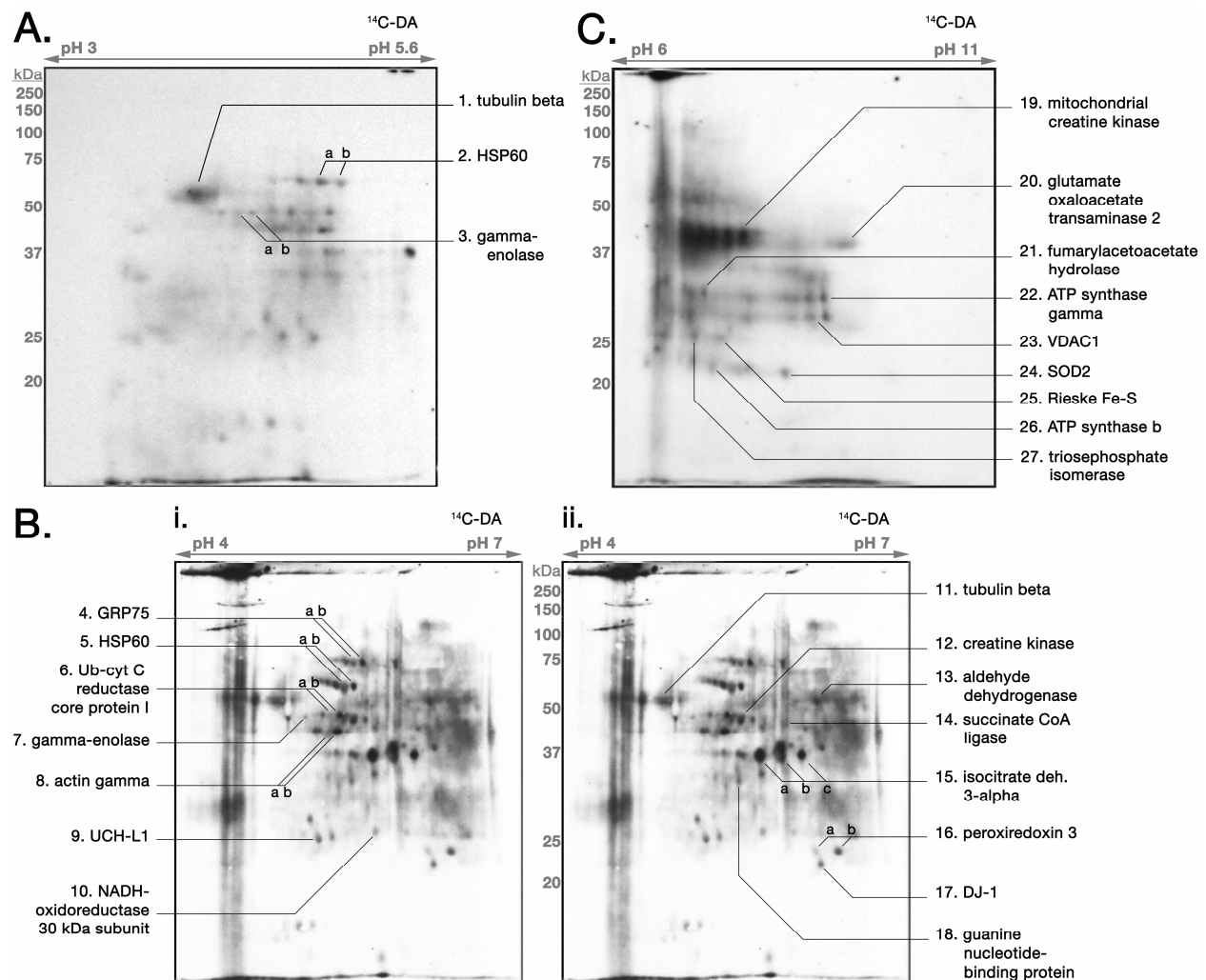


Figure 7: ^{14}C -DAQ Exposure of Isolated Brain Mitochondria Results in DA Conjugation to Select Mitochondrial Proteins

Isolated rat brain mitochondria were exposed to ^{14}C -DA/tyrosinase (150 μM , 0.5-1 μCi ; 300 U/mL) and subjected to 2-D gel electrophoresis, followed by transblotting to PVDF membrane and autoradiography. Samples were analyzed at pH ranges of (A) pH 3-5.6, (B) pH 4-7, and (C) pH 6-11. Select radioactive spots displaying protein conjugated with ^{14}C -DA were picked from the PVDF membrane and subjected to MS analysis for protein identification. Protein identities are provided for each pI range. The autoradiographic image of a representative pH 4-7 2-D gel protein blot was duplicated in (B-i.) and (B-ii.) for ease of accurate labeling of identified proteins.

Table 2: ¹⁴C-DA-Conjugated Proteins Identified from Rat Brain Mitochondrial Fractions

pH 3-5.6

MS Data:

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Peptide Count
3a. gamma enolase	Gamma-enolase (EC 4.2.1.11) (2-phospho-D-glyceratehydro-lyase) (Neural enolase)	P07323 SwissProt	47110.9	5.03	227	100	25
3b. gamma enolase	Gamma-enolase (EC 4.2.1.11) (2-phospho-D-glyceratehydro-lyase) (Neural enolase)	P07323 SwissProt	47110.9	5.03	123	100	17
2b. HSP60	unnamed protein product [Rattus norvegicus]	gi 1334284 NCBI	57889.7	5.35	130	100	18

Combined MS & MS-MS Data:

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Total Ion Score ^b	Total Ion C.I.% ^b	Peptide Count
2a. HSP60	unnamed protein product [Rattus norvegicus]	gi 1334284 NCBI	57889.7	5.35	325	100	125	100	25
1. Tubulin beta	Tubulin beta chain (T beta-15)	P04691 SwissProt	49931	4.79	82	100	13	94.581	12

pH 4-7

MS Data:

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Peptide Count
8b. actin gamma	PREDICTED: similar to Actin, cytoplasmic 2(Gamma-actin) [Rattus norvegicus]	gi 109492380 NCBI	58670	5.67	81	99.965	12
12. creatine kinase	Creatine kinase B-type (Creatine kinase B chain)	gi 122065316 NCBI	42725.27	5.39	146	100	16
17. DJ-1	DJ-1 protein [Rattus norvegicus]	gi 16924002 NCBI	20189.5	6.32	116	100	11
7. gamma-enolase	phosphopyruvate hydratase (EC 4.2.1.11) - rat	gi 1363309 NCBI	47495.1	5.07	85	99.977	12
4a. GRP75	grp75 [Rattus sp.]	gi 1000439 NCBI	73698.8	5.87	141	100	19
4b. GRP75	grp75 [Rattus sp.]	gi 1000439 NCBI	73698.8	5.87	95	100	14
18. guanine nucleotide-binding protein *	Guanine nucleotide-binding protein G(I)/G(S)/G(T)subunit beta 2(Transducin beta chain 2)	P54313 SwissProt	37331	5.6	56	98.562	9
5a. HSP60	unnamed protein product [Rattus norvegicus]	gi 1334284 NCBI	57889.7	5.35	217	100	25
5b. HSP60	unnamed protein product [Rattus norvegicus]	gi 1334284 NCBI	57889.7	5.35	137	100	19
15a. isocitrate dehydrogenase	isocitrate dehydrogenase 3 (NAD+) alpha [Rattusnorvegicus]	gi 16758446 NCBI	40044.2	6.47	189	100	22
15b. isocitrate dehydrogenase	isocitrate dehydrogenase 3 (NAD+) alpha [Rattusnorvegicus]	gi 16758446 NCBI	40044.2	6.47	148	100	18
11. tubulin beta	Tubulin beta-2A chain (T beta-15) - Rattus norvegicus (Rat)	P85108 SwissProt	49907	4.78	226	100	25
6b. Ub-cyt C core protein 1	Ubiquinol-cytochrome-c reductase complex core protein1, mitochondrial precursor	Q68FY0 SwissProt	53499.7	5.57	83	99.997	12
9. UCH-L1	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (EC3.4.19.12) (UCH-L1)	Q00981 SwissProt	25164.6	5.14	80	99.994	9

Table 2 (continued)

**pH 4-7
Combined MS & MS-MS Data:**

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Total Ion Score ^b	Total Ion C.I. % ^b	Peptide Count
8a. actin gamma	PREDICTED: similar to Actin, cytoplasmic 2(Gamma-actin) [Rattus norvegicus]	gi 109492380 NCBI	58763.5	5.67	252	100	184	100	12
13. aldehyde dehydrogenase	mitochondrial aldehyde dehydrogenase precursor [Rattus norvegicus]	gi 45737866 NCBI	56079.4	6.69	318	100	200	100	16
15c. isocitrate dehydrogenase	isocitrate dehydrogenase 3 (NAD+) alpha [Rattusnorvegicus]	gi 16758446 NCBI	40044.2	6.47	277	100	190	100	12
10. NADH-oxidoreductase 30 kDa subunit	NADH dehydrogenase (ubiquinone) Fe-S protein 3(predicted), isoform CRA_b [Rattus norvegicus]	gi 149022594 NCBI	34391.7	8.67	133	100	37	97.67	13
16a. peroxiredoxin 3	Thioredoxin-dependent peroxide reductase, mitochondrial precursor (Peroxiredoxin-3) (PRX-3)	gi 118597399 NCBI	28562.6	7.14	248	100	191	100	7
16b. peroxiredoxin 3	Thioredoxin-dependent peroxide reductase, mitochondrial precursor - Rattus norvegicus (Rat)	Q9Z0V6 SwissProt	28562.6	7.14	91	100	77	100	3
14. succinate CoA ligase	PREDICTED: similar to succinate-Coenzyme A ligase,ADP-forming, beta subunit [Rattus norvegicus]	gi 62661722 NCBI	50616.3	7.57	232	100	130	100	15
6a. Ub-cyt C core protein 1	ubiquinol-cytochrome c reductase core protein I [Rattus norvegicus]	gi 51948476 NCBI	53499.7	5.57	364	100	229	100	16

**pH 6-11
MS Data:**

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Peptide Count
26. ATP synthase b *	ATP synthase B chain, mitochondrial	Q9CQQ7 SwissProt	28930.4	9.11	50	99.06	10
20. glutamate oxaloacetate transaminase 2	glutamate oxaloacetate transaminase 2 [Rattusnorvegicus]	gi 6980972 NCBI	47683.2	9.13	181	100	19
27. triosephosphate isomerase	Triosephosphate isomerase (EC 5.3.1.1)	P48500 SwissProt	26772.7	6.51	44	98.941	7
24. SOD2 *	Superoxide dismutase [Mn], mitochondrial precursor (EC 1.15.1.1) - Rattus norvegicus	P07895 SwissProt	24658.6	8.96	59	99.327	7
23. VDAC1 *	Unknown (protein for IMAGE:7462139) [Rattusnorvegicus]	gi 75773332 NCBI	31920.2	8.35	87	99.993	10

Combined MS & MS-MS Data:

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Total Ion Score ^b	Total Ion C.I. % ^b	Peptide Count
22. ATP synthase gamma	ATP synthase gamma chain, mitochondrial - Rattusnorvegicus (Rat)	P35435 SwissProt	30228.7	8.87	54	97.427	25	98.625	6
20. fumarylacetoacetate hydrolase	PREDICTED: similar to fumarylacetoacetate hydrolasedomain containing 2A [Rattus norvegicus]	gi 34858672 NCBI	40884.2	8.49	105	100	55	99.989	8
19. mitochondrial creatine kinase	creatine kinase, mitochondrial 1, ubiquitous [Rattusnorvegicus]	gi 60678254 NCBI	46932.2	8.58	175	100	70	99.998	15
25. Rieske Fe-S	Rieske Fe-S protein precursor	gi 206681 NCBI	27671.3	8.9	119	100	74	100	7

Notes for Table 2:

a = Protein Spots correspond with Figure 7

b = Probability-based MOWSE score (Protein Score), Protein Score Confidence Interval (C.I.), Total Ion Score, and Total Ion C.I.% represent the top score and C.I. pairing obtained across all blots from which the protein was confidently identified

* = Identified by MS-based peptide mass fingerprinting in only one blot, but corresponding spots were observed in blots from 2 or more separate experiments
For all other identities provided, n = 2-4 for MS analyses, n = 1-3 for MS/MS analyses

4.4.2 DA conjugated proteins can be identified by comparing 2-D DIGE Fluorescent Dye Labeling and ^{14}C -DA autoradiography

Previously, utilizing 2-D DIGE and MS techniques, we demonstrated that *in vitro* exposure of rat brain mitochondria to DAQ resulted in decreased abundance of several identified mitochondrial proteins (Van Laar et al., 2008; see **Chapter 3**). We reasoned here that by combining 2-D DIGE fluorescent labeling technology with autoradiographic techniques, we could take advantage of the protein identification map we already established for 2-D DIGE analysis of rat brain mitochondria. Not only would this methodology provide possible identities of covalently modified proteins, but would also inform us as to whether covalent modification by DA correlated with the loss of abundance in select mitochondrial proteins following DAQ exposure.

Cysteine CyDye DIGE gels of rat brain mitochondria (Cys-CyDye Cy5 control vs. Cys-CyDye Cy3 ^{14}C -DAQ-exposed protein; **Figure 8A**) demonstrated a spot patterning and differential fluorescence identical to that which we previously described (Van Laar et al., 2008; see **Chapter 3**), as did the Lysine CyDye labeled DIGE gels of ^{14}C -DAQ-exposed protein (data not shown). DIGE gels of ^{14}C -DA-exposed mitochondrial proteins (Cys-CyDye DIGE, $n = 5$ gels from 5 separate experiments; Lys-CyDye DIGE, $n = 4$ gels from 3 separate experiments) were imaged and then transblotted to a membrane for autoradiography. The resulting autoradiogram was then compared back to the parent gels. **Figure 8B** shows the autoradiogram generated from a blot of the representative ^{14}C -DA Cys-CyDye DIGE gel in **Figure 8A**, and **Figure 8C** shows the digital merge of the corresponding fluorescent and autoradiographic images.

The identities of several proteins associated with ^{14}C -DA labeling were determined based on identities established for analogous spots from our previous DIGE experiments (Van Laar et al., 2008; see **Chapter 3**) (**Figure 8C; bold text**). Several proteins that we previously identified as decreased in abundance following exposure of brain mitochondria to DAQ were also found to be covalently modified by ^{14}C -DAQ (**Figure 8C; marked with ***). These proteins include mitofilin, 75kDa subunit of Complex I, mortalin/GRP75/mtHSP70, fumarylacetoacetate hydrolase domain protein, superoxide dismutase 2 (SOD2), MtCK, and the TCA cycle proteins isocitrate dehydrogenase 3-alpha subunit and succinate-CoA ligase. ^{14}C -DA modification was also observed correlating with several protein spots whose abundances do not appear to be altered following DAQ exposure. The chaperone HSP60 and TCA cycle protein aconitase, which we identified previously but were not decreased in abundance (Van Laar et al., 2008; see **Chapter 3**), were found to be covalently modified (**Figure 8C**).

We also observed several ^{14}C -DA conjugated protein spots that did not appear decreased in abundance, which were not previously identified in our other DIGE experiments. To identify these proteins, Cys- and Lys-CyDye 2-D DIGE gels with control and non-radiolabeled DA exposed mitochondrial protein were run in parallel with the ^{14}C -DAQ DIGE gels, as described in the methods. Protein spots identified in these experiments as corresponding with covalent ^{14}C -DA modification include actin, ubiquinol cytochrome-C reductase core protein 1, tubulin, and UCH-L1 (**Figure 8C, Table 3**). DeCyder analysis confirmed that the overall normalized abundance of these proteins does not decrease following DAQ exposure (data not shown).

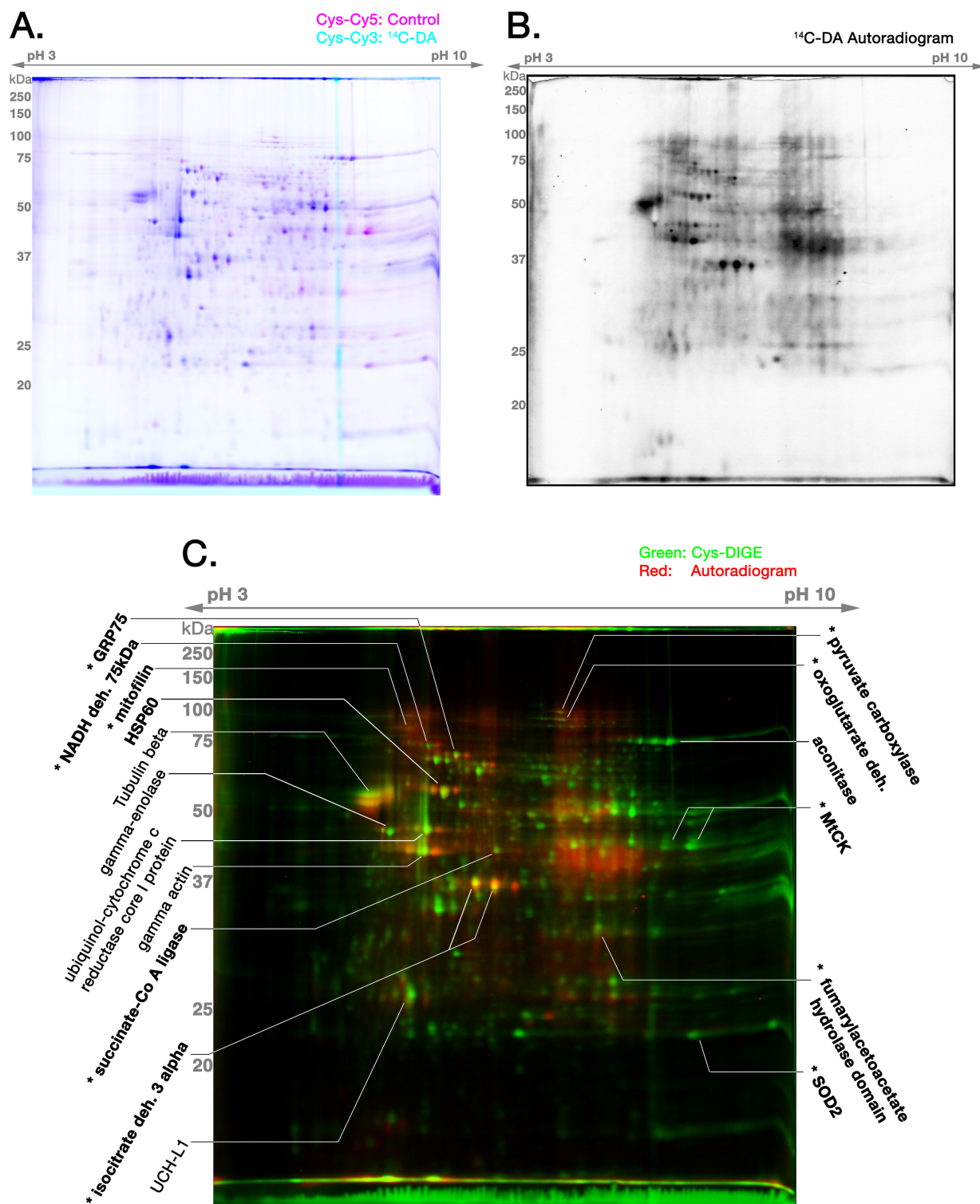


Figure 8: ^{14}C -DAQ Exposure of Isolated Brain Mitochondria Combined with 2-D DIGE

Figure 8 (continued): ^{14}C -DAQ Exposure of Isolated Brain Mitochondria Combined with 2-D DIGE

Lysed Control (C) and ^{14}C -DAQ exposed (^{14}C -DA) mitochondrial proteins were reacted separately with a minimal concentration of cysteine-reactive CyDyes, and then analyzed by 2-D DIGE and autoradiography. (A) Cysteine-labeled (maleimide dye) DIGE gel of Control mitochondrial protein (Cy5, **magenta**) and ^{14}C -DAQ exposed mitochondrial protein (Cy3, **cyan**). (B) Autoradiogram generated by the PVDF transblot of the ^{14}C -DIGE gel shown in (A). (C) Merged pseudocolor image of the fluorescent cysteine-labeled ^{14}C -DA DIGE (Cys-CyDye DIGE) image in (A) (**green**) and its corresponding autoradiogram of the PVDF transblot (**red**). **Yellow** spots demonstrate proteins visualized by DIGE that are conjugated to ^{14}C -DA. Protein identifications were obtained from parallel non-radioactive DIGE gels treated identically to ^{14}C -DA DIGE experiments. **BOLD** protein identifications were previously described (Van Laar et al., 2008; see **Chapter 3**). * indicates proteins which were also found to be decreased in abundance following DAQ exposure based on previous DeCyder analysis of DIGE gels (Van Laar et al., 2008; see **Chapter 3**).

Table 3: Identified Proteins from Control vs DA DIGE Corresponding with Radiolabeling

Protein Spot ^a	Protein Identification	Accession Number & Database	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Peptide Count
tubulin beta	Tubulin, beta, 2 [Rattus norvegicus]	gi 38014578 NCBI	49769	4.79	225	100	24
gamma enolase	enol_cds [Rattus norvegicus]	gi 1619609 NCBI	47110.9	5.03	139	100	19
Ub-cyt C red core 1 protein	Ubiquinol-cytochrome c reductase core protein 1 [Rattusnorvegicus]	gi 51259340 NCBI	52815.4	5.57	91	99.997	14
actin gamma	similar to gamma actin-like protein [Rattus norvegicus]	gi 34875636 NCBI	43082.3	5.11	143	100	17
UCH-L1	Ubiquitin carboxy-terminal hydrolase L1 [Mus musculus] (Protein Group: ubiquitin carboxyl-terminal hydrolase PGP9.5 Rattus)	gi 25058057 NCBI	25164.6	5.14	87	99.996	9

Notes for Table 3:

a = Protein Spots correspond with Figure 8

b = Probability-based MOWSE score (Protein Score) and Protein Score Confidence Interval (C.I.) represent the top score and C.I. pairing obtained across all blots from which the protein was confidently identified ($n = 3-5$)

4.4.3 Comparing ^{14}C -DA autoradiography, 2-D DIGE, and Western blot detection demonstrates DA conjugation with MtCK and mitofilin.

Following autoradiography, blots of ^{14}C -DA 2-D DIGE gels were used for immunochemical detection of MtCK and mitofilin. Both proteins were previously identified from Cys- and Lys-CyDye DIGE gels comparing DAQ-exposed and control rat brain mitochondria (Van Laar et al., 2008; see **Chapter 3**). Here, Western blot analysis confirmed the positions of MtCK (**Figure 9**) and mitofilin (**Figure 10**) within the ^{14}C -DA 2-D DIGE gel blots. Alignment of digital images of the parent fluorescent gels and the resulting autoradiograms demonstrated the association of covalently bound ^{14}C -DA with these two proteins (**Figures 9D** and **10D**).

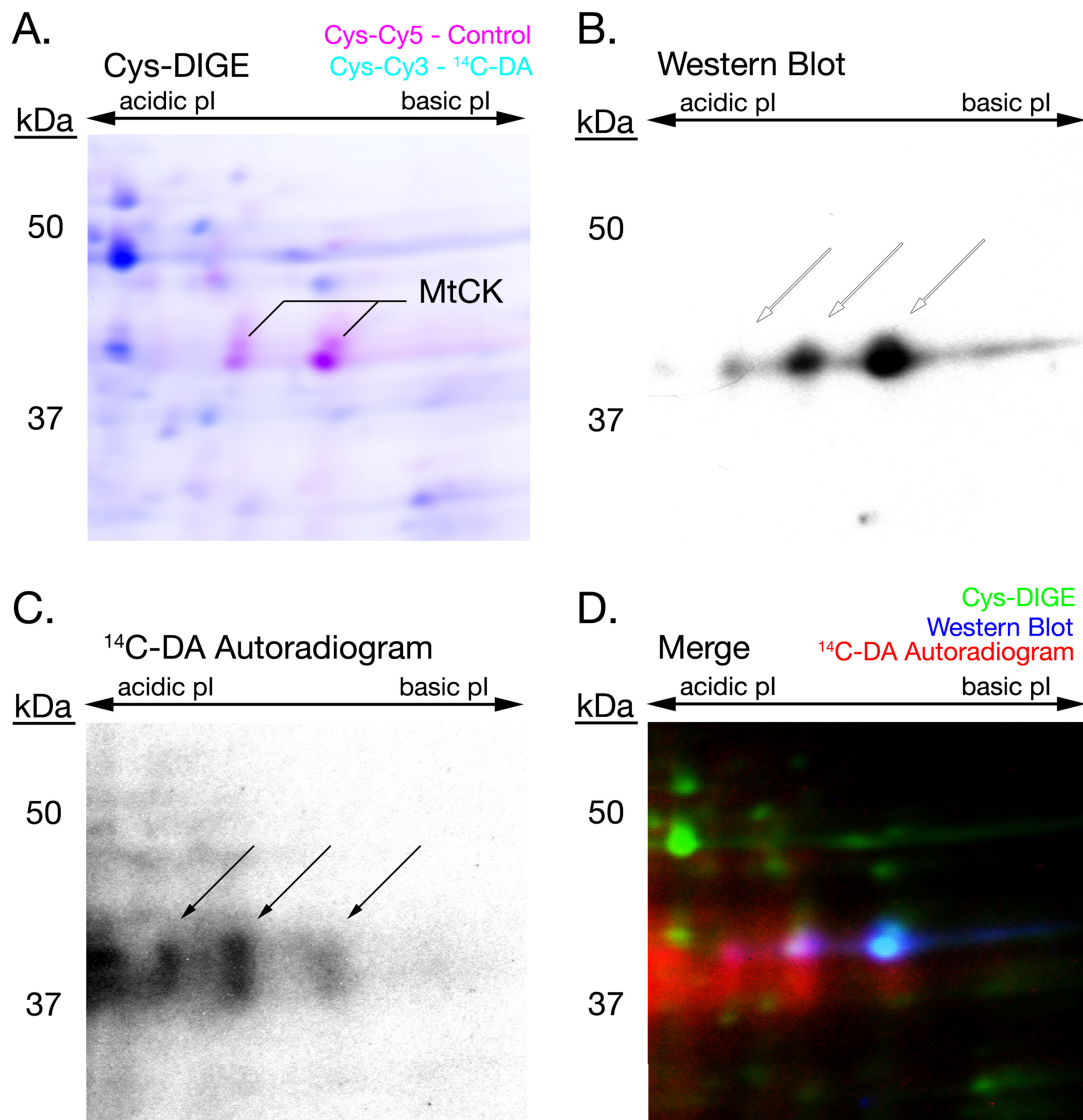


Figure 9: ^{14}C -DA Conjugation with MtCK

A ^{14}C -DA Cys-CyDye DIGE gel was transferred to PVDF membrane, followed by autoradiography and Western blot analysis to detect MtCK. The fluorescent scan of the DIGE gel (A), Western blot (B), and autoradiogram (C) of ^{14}C -DA-labeled mitochondrial creatine kinase (MtCK) were merged (D) to demonstrate the association between ^{14}C -DA-modification (red), immunodetection (blue), and the known DIGE analysis location (green) of MtCK. Arrows point to the location of MtCK in (B) and (C).

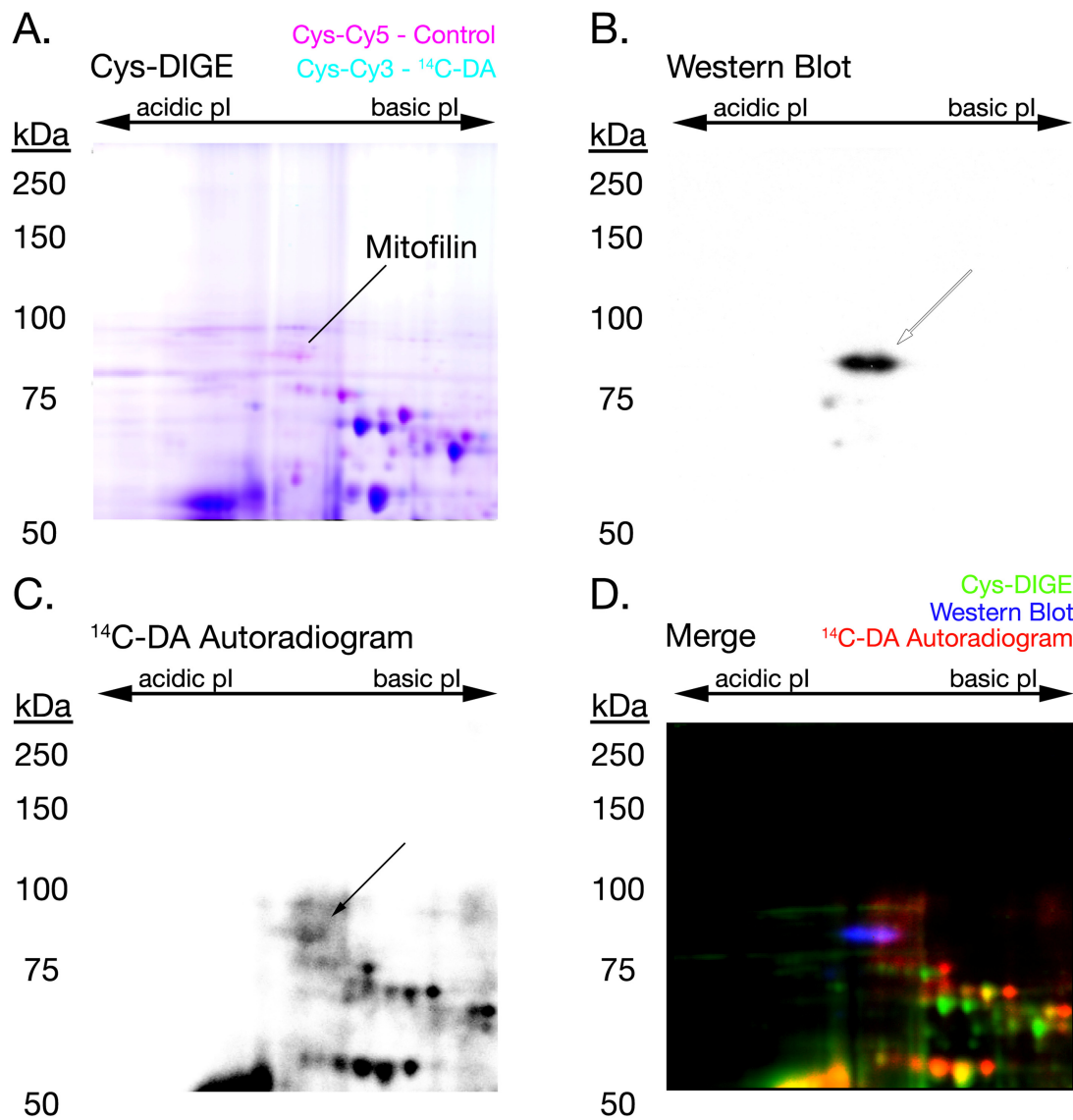


Figure 10: ^{14}C -DA Conjugation with Mitofilin

A ^{14}C -DA Cys-CyDye DIGE gel was transferred to PVDF membrane, followed by autoradiography and Western blot analysis to detect mitofilin. The fluorescent scan of the DIGE gel (A), Western blot (B), and autoradiogram (C) of ^{14}C -DA-labeled mitochondrial mitofilin were merged (D) to demonstrate the association between ^{14}C -DA-modification (red), immunodetection (blue), and the known DIGE analysis location (green) of mitofilin. Arrows point to the location of mitofilin in (B) and (C).

4.4.4 ^{14}C -DA-conjugated proteins were identified from differentiated SH-SY5Y cells.

Differentiated SH-SY5Y cells were exposed to $150\ \mu\text{M}$ ^{14}C -DA ($1\ \mu\text{Ci/mL}$ media) for 16 hrs, a concentration and time point of toxicity established in our laboratory to induce slight ($\sim 10\%$) yet significant cell death (unpublished data). Whole cell lysates from three separate platings of cells with ^{14}C -DA treatment were independently separated by 2-D gel electrophoresis, and the resulting blots of the gels were used to generate autoradiograms to map DA-conjugated proteins. A representative autoradiogram is presented in **Figure 11**. We found that a subset of cellular proteins was covalently modified following SH-SY5Y cell exposure to exogenous radiolabeled DA (**Figure 11**). Using MS and MS + MS/MS analyses, we identified several ^{14}C -DA conjugated proteins (**Figure 11; Table 4**). These proteins include peroxiredoxin 2 isoform A, nucleoside diphosphate kinase A, superoxide dismutase 1 (SOD1), ER-60, mortalin/GRP75/mtHSP70, tropomyosin, UCH-L1, and DJ-1. Note that two protein identities, actin and chloride intracellular channel protein, were confirmed by MS peptide mass fingerprinting analysis in only one blot, though corresponding radiolabeled spots were present in blots from all three experiments. Interestingly, some of the modified proteins identified in SH-SY5Y cells, actin, DJ-1, UCH-L1, and mortalin/GRP75/mtHSP70, were also detected as covalently modified by ^{14}C -DA in the rat brain mitochondrial model of DAQ exposure.

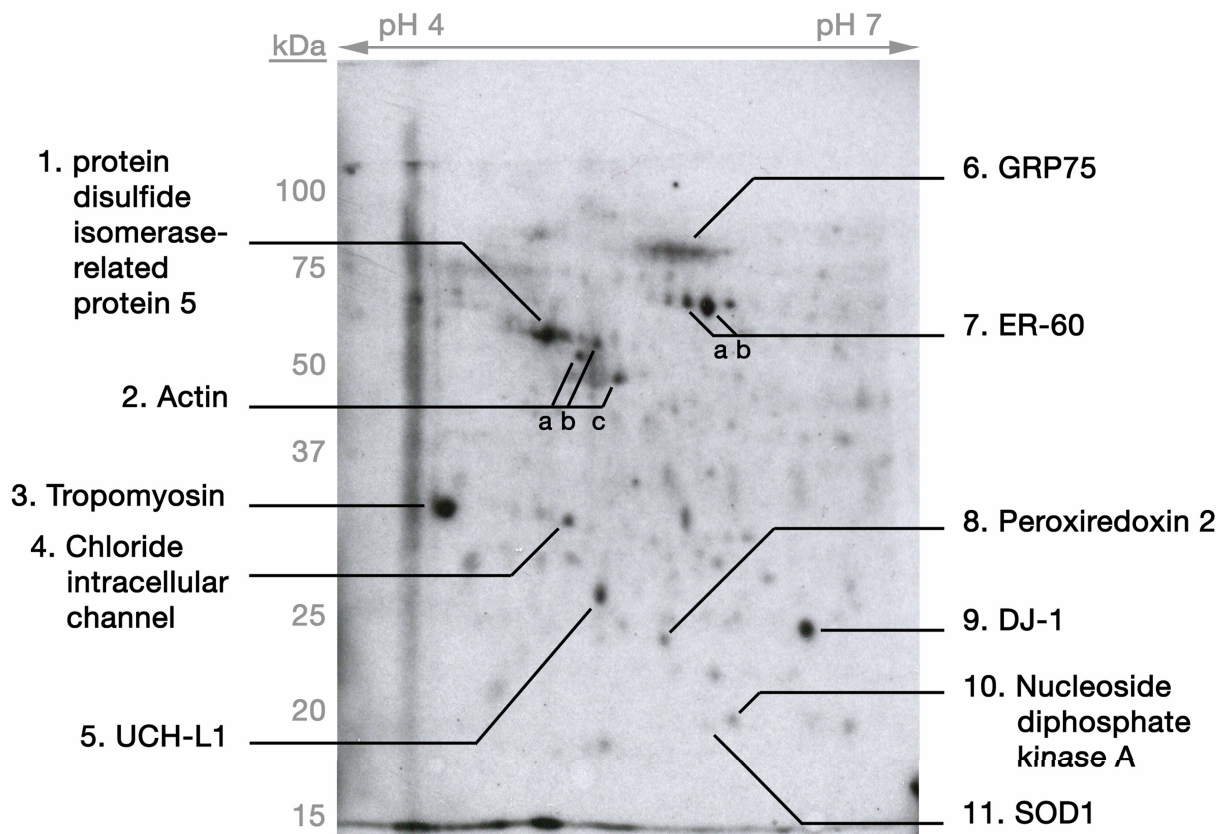


Figure 11: Exposure of Differentiated SH-SY5Y Cells to Exogenous ^{14}C -DA Results in DA Conjugation to Cellular Proteins

Differentiated SH-SY5Y cells were treated with ^{14}C -DA (150 μM ; 10 $\mu\text{Ci/mL}$ media) and subjected to 2-D gel electrophoresis, followed by transblotting to PVDF membrane and autoradiography. Samples were analyzed at a pH ranges of 4-7 pI. Select radioactive spots displaying protein conjugated with ^{14}C -DA were picked from the PVDF membrane and subjected to MS analysis for protein identification. Protein identities are indicated on autradiogram.

Table 4: Identified DA-Conjugated Proteins from ¹⁴C-DA-Exposed SH-SY5Y Cells

Table 3. Identified DA-Conjugated Proteins from DA-Exposed SH-SY5Y Cells

MS Data:

Protein Spot ^a	Protein Identification	Accession Number	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Peptide Count
2a. Actin *	ACTB protein [Homo sapiens]	gi 15277503	40194	5.55	108	100	15
2b. Actin *	ACTB protein [Homo sapiens]	gi 15277503	40194	5.55	146	100	16
2c. Actin *	actin, gamma 1 propeptide [Homo sapiens]	gi 4501887	41662	5.31	138	100	17
4. Chloride intracellular channel*	chloride intracellular channel 1 [Homo sapiens]	gi 14251209	26792	5.09	119	100	13
7a. ER-60	protein disulfide isomerase family A, member 3, isoformCRA_a [Homo sapiens]	gi 119597640	54454	6.78	249	100	28
1. protein disulfide isomerase related protein 5 *	protein disulfide isomerase-related protein 5 [Homo sapiens]	gi 1710248	46170.2	4.95	131	100	28
3. Tropomyosin	hypothetical protein [Homo sapiens]	gi 57997573	27387	4.71	199	100	22
5. UCH-L1	ubiquitin carboxyl-terminal esterase L1	gi 21361091	25151	5.33	148	100	15

Combined MS & MS-MS Data:

Protein Spot ^a	Protein Identification	Accession Number	Theoretical Protein MW	Theoretical Protein pI	Protein Score ^b	Protein Score C.I.% ^b	Total Ion Score ^b	Total Ion C.I.% ^b	Peptide Count
6. GRP75	heat shock 70kDa protein 9B precursor	gi 24234688	73920	5.87	145	100	94	100	10
7b. ER-60	protein disulfide isomerase [Homo sapiens]	gi 860986	57043	6.1	393	100	210	100	21
9. DJ-1	(Q99497) Protein DJ-1 (Oncogene DJ1)	Q99497 **	19879	6.33	172	100	78	100	10
8. Peroxiredoxin 2	TSA [Homo sapiens]	gi 1617118	18486	5.19	325	100	236	100	9
10. Nucleoside diphosphate kinase	NME1-NME2 protein [Homo sapiens]	gi 66392203	30346	9.06	228	100	147	100	8
11. SOD1	Chain A, A4v Mutant Of Human Sod1	gi 47169370	16051	5.7	95	99.993	52	99.86	4

Notes for Table 3:

a = Protein Spots correspond with Figure 11

b = Probability-based MOWSE score (Protein Score) Protein Score Confidence Interval (C.I.) and Total Ion Score and Total Ion C.I.% represent the top score and C.I. pairing obtained across all blots from which the protein was confidently identified

* = Identified by MS-based peptide mass fingerprinting in only one blot, but corresponding spots were observed in blots from 2 or more separate experiments
For all other identities provided, *n* = 2-4 for MS analyses, *n* = 1-3 for MS/MS analyses

**= Accession Number from SwissProt database. All others are from NCBI database.

4.5 DISCUSSION

4.5.1 Summary

Oxidative damage of proteins has been associated with the pathogenesis of various neurodegenerative diseases, including Alzheimer's disease and PD (Halliwell, 2001; Halliwell, 2006). Proteomic studies have identified multiple protein targets of oxidation in association with these diseases (Castegna et al., 2002a; Castegna et al., 2002b; Choi et al., 2004b; Choi et al., 2005; Choi et al., 2006). Utilizing a DA oxidation model of PD, we identified protein targets covalently modified by DAQ. Proteomic analysis of isolated rat brain mitochondria exposed to ^{14}C -DAQ *in vitro* revealed a subset of mitochondrial proteins conjugated to DA, including mortalin/GRP75/mtHSP70, HSP60, MtCK, mitofilin, glutamate oxaloacetate transaminase 2, isocitrate dehydrogenase 3 subunit alpha, the ubiquinol-cytochrome c reductase core protein 1 and Rieske Fe-S protein subunits of Complex III, and the 75 kDa and 30 kDa subunits of Complex I. We also identified several DA-conjugated proteins from whole-cell lysate of ^{14}C -DA-exposed differentiated SH-SY5Y cells. Some of the proteins identified from SH-SY5Y cells, including actin, UCH-L1, DJ-1, and mortalin/GRP75/mtHSP70, correlated with the mitochondrial ^{14}C -DAQ-exposure model, suggesting that DA-modified proteins identified from DAQ-exposed mitochondria are also targets of modification in living cells. Covalent modification of these proteins by DA may lead to altered structure or inactivation of function, and may play a role in dopaminergic neuron vulnerability in PD pathogenesis.

We utilized two methods for identification of ^{14}C -DA-modified proteins in the mitochondrial model. First, using MS analyses we carried out direct identification of protein spots conjugated to radiolabeled DA from the PVDF membrane. Second, we compared known

spot patterns from fluorescent-labeled 2-D DIGE gels of radiolabeled protein to the autoradiographic images generated from blots of those same gels. In the second method, identities were determined from MS analyses of protein spots from parallel, non-radioactive DIGE gels. Most of the identified covalently-modified proteins were similar between the two methods, validating both the protein identities and the individual methods for identifying DA-modified proteins.

Noting that isolated brain mitochondria were intact when exposed to ^{14}C -DAQ, it is interesting that we identified covalently-modified proteins associated with the mitochondrial matrix as well as the intermembrane space. This suggests that the electrophilic DAQ gains access to both compartments in the intact mitochondrion at a physiological pH (pH 7.4). This finding also has implications for the reactivity of specific mitochondrial proteins to DA quinone. Thus, the ^{14}C -DA labeled spots observed in this study represent proteins that are the most reactive and/or accessible to DAQ as compared to the remainder of the mitochondrial proteome.

Some proteins we identified from our DAQ-exposed rat brain mitochondrial isolates, such as triosephosphate isomerase and UCH-L1, are not typically associated with the mitochondria. We are aware that the mitochondrial-enriched fractions isolated from brain by our described procedure typically contain approximately 10% of contaminating synaptosomes (Berman et al., 2000). Thus, we are not asserting that such proteins are necessarily mitochondrial. This fact does not, however, make the identification of DA-conjugated non-mitochondrial proteins in these samples any less significant. We demonstrate here that the cytosolic proteins actin, tubulin, UCH-L1, DJ-1, and triosephosphate isomerase in their native state are potential targets of DA modification when exposed to DAQ *in vitro*. Further, actin,

UCH-L1, and DJ-1 were also subject to covalent modification in DA-exposed SH-SY5Y cells, which may have implications for the role of these proteins in neurodegeneration.

4.5.2 Several mitochondrial proteins covalently modified by DA displayed changes in abundance

Several proteins we identified here as being covalently modified by DAQ were also identified in our previous study as being decreased in abundance following mitochondrial exposure to DAQ (Van Laar et al., 2008; see **Chapter 3**). The proteins MtCK and mitofilin, in particular, were previously confirmed to be decreased in DAQ-exposed rat brain mitochondria and DA-exposed PC12 cells via Western blot analysis, and are demonstrated here, by comparison of Western blot and autoradiography, to also be covalently modified by ^{14}C -DA. We previously hypothesized that the loss of protein may involve either rapid aggregation or proteolytic degradation of oxidatively modified proteins. However, other proteins identified as DA-conjugated in this study were not associated with any measurable change abundance, such as HSP60 and aconitase. Thus, covalent modification by DAQ does not necessarily correspond with decreases in protein abundance in mitochondria. This may have implications for the susceptibility of specific mitochondrial proteins for proteolytic degradation or aggregation following oxidative modification. Further study will be necessary to evaluate the differences in individual protein responses to DA oxidation both in mitochondria and in cells.

4.5.3 Protein Targets of DA Conjugation Encompass Multiple Critical Functions, and are Known Targets for Oxidative Modification

The protein targets of covalent modification by DAQ that we have identified are involved in a range of critical mitochondrial and cellular functions. Because they are susceptible to modification by the electrophilic DAQ, many of the proteins identified in this study are likely vulnerable to other oxidative agents that induce modification. Indeed, several proteins identified here have been reported in other studies to be oxidatively modified in association with disease, disease models, and oxidative stress. One such protein is mitofilin, which we previously identified as exhibiting decreased abundance following DAQ-exposure (Van Laar et al., 2008; see **Chapter 3**). Various studies have shown that mitofilin is susceptible to oxidative stress, demonstrating oxidatively-modified cysteine residues following alcohol exposure in hepatoma cells (Suh et al., 2004), as well as a ROS-induced reduction of protein levels (Magi et al., 2004). Given the proposed role of mitofilin in maintaining mitochondrial cristae morphology (John et al., 2005) and interaction with key mitochondrial import proteins (Xie et al., 2007), oxidative modification could have a detrimental impact on proper function or protein-protein interactions, and thus on mitochondrial stability.

Protein import and processing could also be impacted by the modification of two mitochondrial protein chaperones, HSP60 and mortalin/GRP75/mtHSP70, as identified in this study. HSP60 and mortalin/GRP75/mtHSP70 are key protein processing chaperones in the mitochondria, with roles in matrix protein folding and mitochondrial protein import, respectively (Wadhwa et al., 2005; Yaguchi et al., 2007). Mortalin/GRP75/mtHSP70, in particular, is a known target of oxidative stress, and has previously been linked to neurodegenerative diseases PD and AD based on altered expression (Jin et al., 2006; Osorio et al., 2007). A recent study

found that both HSP60 and Mortalin/GRP75/mtHSP70 interacted with frataxin, a protein involved in iron-sulfur (Fe-S) cluster biogenesis for Fe-S cluster-dependent enzymes (Shan et al., 2007). The authors also noted that Mortalin/GRP75/mtHSP70 shares homology with the HSP70-family protein Ssq1, a mitochondrial matrix protein required for Fe-S cluster assembly (Lutz et al., 2001; Shan et al., 2007). Interestingly, we identified several Fe-S cluster-containing proteins covalently modified by ^{14}C -DA, including the 30 kDa subunit of Complex I, Rieske Fe-S protein subunit of Complex III, and aconitase. Thus, DA oxidation may potentially contribute to an impaired Fe-S protein system in dopaminergic neurons, both in Fe-S protein biogenesis and function.

Multiple subunits of Complex I (75 kDa and 30 kDa subunits) and Complex III (ubiquinol-cytochrome c reductase core protein 1 and Rieske Fe-S protein) of the mitochondrial electron transport chain (ETC) were identified as targets of covalent DA modification. Several studies have demonstrated that incubation of isolated brain mitochondria with DA or DAQ inhibits mitochondrial respiration (Berman and Hastings, 1999; Cohen et al., 1997; Gluck et al., 2002; Gluck and Zeevalk, 2004). Studies in disrupted mitochondria have also suggested DA can directly interact with and inhibit Complex I (Ben-Shachar et al., 2004; Brenner-Lavie et al., 2008). Decreased NADH dehydrogenase activity of Complex I has been observed in both the SN (Janetzky et al., 1994; Orth and Schapira, 2002; Schapira et al., 1990) and the periphery (Blandini et al., 1998; Shoffner et al., 1991) of PD patients. PD models utilizing Complex I inhibitors rotenone and 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine (MPTP) replicate characteristics of the disease, including nigrostriatal dopaminergic cell death (Przedborski et al., 2000; Sherer et al., 2003a). Modification of critical ETC proteins by DA oxidation may

potentially inhibit these proteins, possibly leading to an increase in mitochondrial dysfunction and play a role in the increased susceptibility of dopaminergic neurons in PD.

In this study, we also found both cytosolic and mitochondrial isoforms of CK to be targets of DA modification in isolated brain mitochondria. Previous studies have shown that exposure to dopamine and dopamine oxidation can inhibit the activity of CK proteins (Maker et al., 1986; Miura et al., 1999). Choi et al. also found that cytosolic creatine kinase (CK) was susceptible to increased oxidation in the hippocampus of aged mice, as well as in young and old ApoE-KO mice, as compared to brain tissue of young mice (Choi et al., 2004a). CK was also identified as having increased carbonyl modification in AD brain (Castegna et al., 2002a). Given the critical role of CK and MtCK in ATP level maintenance (Eder et al., 2000), and the association of MtCK with the proteins involved in the permeability transition pore (Vyssokikh and Brdiczka, 2003), oxidative modification of CK proteins may carry implications for energy maintenance and mitochondrial function in dopaminergic neurons. In a similar vein, we found ¹⁴C-DA covalent modification of the protein nucleoside diphosphate kinase A in SH-SY5Y cells. Considered a multifunctional protein, the assigned function of this enzyme is to maintain a balance between ADP, GDP, ATP, and GTP levels in the cell, and its activity is known to be modulated by disulfide crosslinking of two oxidation-sensitive cysteine residues (Cumming et al., 2004; Song et al., 2000).

Though not necessarily mitochondrial, the glycolysis proteins enolase 2 and triosephosphate isomerase were also shown in this study to be modified by DA in DAQ-exposed mitochondria preparations. These proteins have previously been shown to be targets of oxidative modification in association with neurodegenerative disease. Triosephosphate isomerase, alpha- and gamma-enolase, and beta-actin were found to be targets of protein nitration in AD brain

(Castegna et al., 2003). Oxidative modification of these proteins by DAQ could have implications for dysfunction in cellular metabolism and energy generation upstream of the ETC, which could have detrimental effects for the high-energy demands of neurons.

4.5.4 Antioxidant and Thiol Oxidoreductase Enzymes are covalently modified by DAQ

We observed covalent DA modification of mitochondrial SOD2 and peroxiredoxin 3 in ¹⁴C-DAQ-exposed brain mitochondria, and cytosolic SOD1 and peroxiredoxin 2 in ¹⁴C-DAQ-exposed differentiated SH-SY5Y cells. Both enzyme types are integral in managing ROS levels and protecting against oxidative stress. SOD enzymes catalyze conversion of the free radical superoxide to oxygen and H₂O₂, and peroxiredoxins catalyze reduction of H₂O₂ to water. Alterations in expression and activity of both enzymes have been linked to PD and PD models. Post mortem PD brain tissue displayed increased SOD activity (Saggu et al., 1989), and elevated levels of SOD2 have been observed in PD patient CSF (Yoshida et al., 1994), suggestive of a response to increased levels of superoxide. Peroxiredoxin 2, which is highly abundant in neurons, showed increased abundance in PD brain SN (Basso et al., 2004), again suggestive of an oxidative stress response. Peroxiredoxin 2 was also found to be S-nitrosylated in rotenone and MPP⁺ treated SH-SY5Y cells and in PD brain (Fang et al., 2007). In addition, silencing expression of mitochondrial peroxiredoxin 3 and 5 increased the vulnerability of SH-SY5Y cells to MPP⁺ toxicity (De Simoni et al., 2008). Thus, DA modification of these enzymes could compromise the antioxidant defense mechanisms of dopaminergic neurons.

In this study, we also observed DA modification of the endoplasmic reticulum chaperones ER-60 (also known as GRP58 or ERp57) and protein disulfide isomerase-related protein 5 (also known as ERp5 or PDIA6) in SH-SY5Y cells exposed to DA. Both proteins are

members of the protein disulfide isomerase family, which employ thiol-disulfide oxidoreductase activity to oxidize disulfide bonds in substrates, and thus mediate proper protein folding (Ellgaard and Ruddock, 2005). Both proteins are primarily localized to the endoplasmic reticulum, and each contains two cysteine-glycine-histidine-cysteine, or C-X-X-C, thioredoxin-like activity motifs integral in enzymatic disulfide bond formation (Ellgaard and Ruddock, 2005). Proteins containing the C-X-X-C motif are very susceptible to modification by electrophilic compounds such as DAQ (Lame et al., 2003; Lame et al., 2005). ER-60 also has demonstrated cysteine protease activity (Okudo et al., 2000; Urade and Kito, 1992; Urade et al., 1992) and a role in degradation of misfolded proteins (Otsu et al., 1995). We have previously shown the upregulation of ER-60 expression along with other ER chaperones following DA exposure in differentiated PC12 cells, suggesting DA toxicity induces ER stress and the unfolded protein response (Dukes et al., 2008). As aberrant protein folding and aggregation are associated with PD, DA may be contributing by modification and inactivation of key ER chaperone proteins.

4.5.5 DJ-1 and UCH-L1 Are Targets for Covalent Modification by DAQ

An intriguing finding of this study was the covalent DA modification of two proteins directly linked to genetic forms of PD, UCH-L1 and DJ-1. DJ-1, linked to familial PARK7 (Bonifati et al., 2003), is found widely distributed in the brain (Bandopadhyay et al., 2004). While the specific function of DJ-1 remains unknown, data suggest a role for the protein in oxidative stress regulation, possibly through the protein's own susceptibility to oxidation of a critical cysteine residue (Canet-Aviles et al., 2004). UCH-L1 is linked to PARK5 through a mutation that leads to a decrease in the deubiquinating activity of the protein (Leroy et al., 1998;

Nishikawa et al., 2003). The exact mechanism by which this mutation leads to decreased activity is unknown. However, it has been shown that the active site of UCH-L1 contains cysteine and histidine residues that are sensitive to modification by 4-hydroxynonenal (4-HNE), a reactive byproduct of lipid peroxidation (Nishikawa et al., 2003). Additionally, oxidative damage of both DJ-1 and UCH-L1, in the form of carbonylation and direct oxidation at cysteines and methionines, has been identified in both PD and AD brain (Castegna et al., 2002a; Choi et al., 2004b; Choi et al., 2006). Thus covalent modification by DAQ may be detrimental to DJ-1 and UCH-L1 function. It is important to note that DA-induced modifications have been associated with alterations in two other PD-linked proteins, alpha-synuclein (Conway et al., 2001) and parkin (LaVoie et al., 2005). The susceptibility of multiple PD-linked proteins to DA modification further supports a contributing role for DA oxidation in dopaminergic neuron degeneration and PD pathogenesis.

4.5.6 DAQ Binds to Cytoskeletal Proteins

We found that cytoskeletal proteins actin and tubulin were targets of covalent modification by DAQ in isolated mitochondria preparations, and actin and interacting protein tropomyosin were covalently modified by DA in DA-exposed differentiated SH-SY5Y cells. Tubulin protein is highly abundant, contains multiple free thiol groups critical for microtubule assembly, and is a known target of oxidative modification (Landino et al., 2002; Luduena and Roach, 1991). Tubulin was modified *in vitro* at specific cysteine residues by 4-HNE (Stewart et al., 2007), which has been shown to disrupt neuronal microtubules and neurite outgrowth in cultured Neuro 2A cells (Neely et al., 2005; Neely et al., 1999). Likewise, beta-actin was observed to be modified by 4-HNE in brain tissue of patients with mild cognitive impairment

patient (Reed et al., 2008). Increased carbonylation and nitration of actin was observed in post mortem AD brain (Aksenov et al., 2001; Castegna et al., 2003). Relevant to our findings, other cytoskeletal proteins have been demonstrated as targets of DA oxidation *in vitro*. Exposure to oxidized DA promoted polymerization of tau protein (Santa-Maria et al., 2005a) and covalent crosslinking of neurofilament proteins (Montine et al., 1995).

It is well known that the transport and localization of mitochondria within the cell relies upon their close interaction with intact cytoskeletal structures (Boldogh and Pon, 2006; Boldogh and Pon, 2007). Thus, oxidation-induced damage to cytoskeletal proteins could have drastic effects on cellular structure, and on critical transport, localization, and ultimately function of organelles such as mitochondria.

4.5.7 Conclusions

In conclusion, we identified proteins in this study that are targets of quinone modification following intracellular DA oxidation, and should be considered for further study in connection with the pathophysiology of PD. The susceptibility of these proteins to modification may play a role in the enhanced vulnerability of DAergic neurons. In addition to protein dysfunction, such oxidative modifications may lead to disrupted protein-protein interactions, targeting of proteins for degradation, or promoting aggregation of damaged proteins, as is observed in PD.

As this is an *in vitro* study, these proteins should be considered for further characterization in *in vivo* models, as well as in PD patients, to evaluate their potential as targets of oxidative stress within the nigrostriatal system. In this regard, it is important to note that many of the proteins we identify here as targets of DA modification have previously been identified as targets of oxidative modification in conjunction with various neurodegenerative

diseases and disease models, as discussed above. The identification of these same proteins across multiple independent studies not only validates their characteristic susceptibility to oxidative modification but also demonstrates their potential significance in neurodegenerative disease pathogenesis. Such proteins deserve critical attention to elucidate their roles in disease progression and their potential as targets for novel therapeutic strategies.

4.6 ACKNOWLEDGMENTS

We thank Dr. Ashraf Elamin, Mirunalni Thangavelu, proteomics manager Dr. Manimalha Balasubramani, and proteomics director Dr. Billy Day of the University of Pittsburgh Genomics and Proteomics Core Laboratories for their excellent technical expertise, advice, and assistance. We also thank and recognize Dr. Amanda Mishizen for her significant contribution to the SH-SY5Y cell culture experiments in this chapter.

5.0 EFFECT OF MITOCHONDRIAL MITOFILIN ON DOPAMINE- AND ROTENONE-INDUCED TOXICITY IN DIFFERENTIATED PC12 AND SH-SY5Y CELLS

5.1 SUMMARY

Mitochondrial dysfunction and oxidative stress have been implicated in the neurodegenerative process in Parkinson's disease. Mitofilin, a protein of the inner mitochondrial membrane, has been shown to be critical for maintaining mitochondrial cristae organization, suggesting an important role in mitochondrial function. Using proteomic techniques, we previously demonstrated that exposure of isolated rat brain mitochondria to dopamine quinone resulted in the loss of a subset of mitochondrial proteins, including mitofilin. However, mitofilin has not been previously characterized in dopaminergic cells. To examine the effect of altering mitofilin levels on dopaminergic cell vulnerability, we utilized overexpression and shRNA knockdown techniques in differentiated PC12 and SH-SY5Y cells. Transient and stable overexpression of FLAG-tagged mouse mitofilin in differentiated PC12 cells significantly attenuated cell death by approximately 50% following dopamine or rotenone exposure, as compared to controls. In differentiated SH-SY5Y cells, transient overexpression of mitofilin significantly attenuated cell death by 60% following DA exposure, and stable overexpression of mitofilin significantly attenuated both dopamine- and rotenone-induced cell death as compared

to treated controls. Conversely, transient knockdown of mitofilin protein in differentiated SH-SY5Y cells potentiated cell death by over 30% following exposure to dopamine as compared to controls. Co-expression of mitofilin protein, not susceptible to the shRNA, rescued the cells from the enhanced vulnerability associated with mitofilin knockdown. These results suggest that altering levels of mitofilin significantly affects the vulnerability of cells to dopaminergic toxins, dopamine and rotenone, carrying implications for Parkinson's disease pathogenesis.

5.2 INTRODUCTION

Mitochondria are the powerhouses of the cell, responsible for regulating various cellular functions and signaling pathways. Maintenance and integrity of mitochondrial structure are essential to cellular health. In particular, organization and shape of the inner membrane, the site of the electron transport chain (ETC) and ATP generation, are suggested to impact mitochondrial function, and may change in response to various factors (for review see Mannella et al., 2001). Dysfunction of mitochondrial respiration can lead to increased oxidative stress and cell death, and has been implicated in the pathogenesis of Parkinson's disease (PD) (Beal, 2007; Schapira, 2008).

Mitochondrial dysfunction may cause oxidative stress, as the mitochondrial ETC is a known source of reactive oxygen species (ROS), and mitochondrial ETC inhibition can lead to increased free radical production (Beal, 2003; Lenaz et al., 2002). Oxidative stress can, in turn, have detrimental effects on general cellular functions and on the mitochondrion itself (Fiskum et al., 2003; Turrens, 2003). Mitochondrial dysfunction is also linked to PD through observations of decreased activity of NADH dehydrogenase (Complex I) in the ETC in both the SN (Janetzky

et al., 1994; Orth and Schapira, 2002; Schapira et al., 1990) and the periphery (Blandini et al., 1998; Shoffner et al., 1991) of PD patients. Chronic administration of rotenone, a high-affinity Complex I inhibitor, to rats resulted in PD-like effects in behavioral disorders and neurodegeneration (Betarbet et al., 2000; Sherer et al., 2003a), supporting a role for Complex I in the pathogenesis of PD. These results suggest a susceptibility of the dopaminergic SN neurons to impaired mitochondrial activity.

Though multiple neuronal types are known to be involved in the symptoms of PD, the unique susceptibility of the nigrostriatal dopaminergic neurons suggests the possibility that dopamine (DA) may be contributing to their degeneration. DA is susceptible to both enzymatic and auto-oxidation, resulting in the formation of reactive DA quinines (DAQ). The Hastings laboratory has previously shown *in vivo* that DA oxidation resulted in covalently modified proteins and the selective loss of DA terminals (Hastings et al., 1996; Rabinovic et al., 2000). Our laboratory and others have also demonstrated that exposure of isolated brain mitochondria to DA and DAQ leads to altered respiration, mitochondrial swelling, and PTP opening (Berman and Hastings, 1999; Cohen et al., 1997; Gluck et al., 2002). We recently demonstrated that exposure of rat brain mitochondria to DAQ results in a significant loss of several mitochondrial proteins (Van Laar et al., 2008). One of the most affected proteins, mitochondrial mitofilin, was decreased 65% following exposure to DAQ (Van Laar et al., 2008). Mitofilin was also found to be decreased in mitochondria isolated from DA-exposed PC12 cells (Van Laar et al., 2008; see **Chapter 3**). Additional proteomic study demonstrated that mitofilin was a target for covalent modification by DAQ (unpublished results; see **Chapter 4**).

The inner mitochondrial membrane protein, mitofilin, first identified in human heart muscle (Icho et al., 1994), is a nuclear-expressed mitochondrial protein that is targeted selectively

to the inner mitochondrial membrane (Gieffers et al., 1997; Odgren et al., 1996). The protein contains a transmembrane domain near the N-terminus that spans the inner mitochondrial membrane, while the bulk of the protein juts into the intermembrane space (Gieffers et al., 1997). Mitofilin also contains three coiled-coil domains (John et al., 2005), which are often involved in protein-protein interactions (Cohen and Parry, 1990). Mitofilin appears to be ubiquitously expressed in all cells containing mitochondria (Gieffers et al., 1997), and is highly conserved across mammals (Odgren et al., 1996; Omori et al., 2002). In humans, the protein is known to have at least two splice variants of 90 and 88 kDa (Gieffers et al., 1997). The specific function of mitofilin remains unknown. However, evidence suggests that the protein is critical in maintaining mitochondrial cristae structure (John et al., 2005).

To elucidate possible functions for mitofilin, John et al. utilized siRNA against mitofilin in cultured HeLa cells (John et al., 2005). A loss of mitofilin resulted in reduced cell growth rate, increased apoptosis, and increased ROS production in HeLa cells (John et al., 2005). Morphologically, a decrease in mitofilin resulted in mitochondria with severely disrupted cristae, suggesting a critical role for mitofilin in mitochondria structural maintenance (John et al., 2005). Recently, mitofilin was found to exist in complexes with proteins integral in mitochondrial protein import, including SAM50 and metaxins 1 and 2 (Xie et al., 2007). These studies suggest that mitofilin and its protein-protein interactions play an important role in normal mitochondrial function and morphology. Thus, a loss or modification of mitofilin could lead to detrimental effects in mitochondrial structure and function. As exposure to DA can alter mitochondrial mitofilin levels in cells, it is possible that alterations in mitofilin protein expression will modulate the vulnerability of dopaminergic cells to toxins that affect mitochondrial function.

In this study, we utilized overexpression and shRNA techniques to examine the effect of modulating mitofilin protein levels on the cellular response to DA- and rotenone-induced toxicity in two dopaminergic cell lines, rat pheochromocytoma PC12 cells and human neuroblastoma SH-SY5Y cells. The results showed that increased mitofilin expression attenuated cell loss following DA or rotenone exposure in both cell types, whereas decreased mitofilin potentiated DA-induced cell death in SH-SY5Y cells. Our findings suggest that altering levels of mitofilin affects the vulnerability of cells to dopaminergic toxins. Given that levels of mitofilin are sensitive to oxidant stressors (Jin et al., 2004; Magi et al., 2004; Van Laar et al., 2008; see **Chapter 3**), these findings may also have implications for mitochondrial dysfunction in PD.

5.3 EXPERIMENTAL PROCEDURES

Materials

Lipofectamine™ 2000, OptiMEM (Gibco), Dulbecco's modified Eagle medium (DMEM; Gibco), fetal bovine serum (FBS; HyClone), horse serum (HS; HyClone), 10,000 U/mL penicillin/10,000 µg/mL streptomycin (pen/strep; Gibco), and Geneticin ® (G418, cat#10131-035; Gibco) were purchased from Invitrogen (Carlsbad, CA). Rat-tail collagen was purchased from BD Bioscience (Bedford, MA), trypsin with 0.25% EDTA from Mediatech (Herndon, VA), nerve growth factor (NGF) from BD Bioscience, and rotenone from MP Biomedicals (Aurora, OH). Dimethyl sulfoxide, retinoic acid, dopamine, Trypan Blue, protease inhibitor cocktail (cat#P2714) and all chemicals for general buffers and solutions were purchased from Sigma (St. Louis, MO), unless otherwise noted.

Antibodies & Plasmids

The polyclonal “Genemed” rabbit anti-mitofilin antibody was made for our laboratory by Genemed Synthesis (San Antonio, TX) (Van Laar et al., 2008; see **Chapter 3**) and used for immunodetection at 1:500 dilution. Mouse anti-FLAG® M2 antibody (used at 1:500 dilution) was purchased from Stratagene (La Jolla, CA). Mouse anti-actin MAB1501 (used at 1:75,000-100,000 dilution) was purchased from Chemicon (Temecula, CA). Rabbit anti-GAPDH ab9485 (used at 1:15,000) was purchased from Novus (Littleton, CO). Fluorescent-conjugated secondary antibodies, goat anti-rabbit IRDye® 800 and goat anti-mouse IRDye® 680, were purchased from Li-Cor Biosciences (Lincoln, NE) and used at 1:10,000 dilution. The “T3867” polyclonal rabbit anti-mitofilin antibody (1:1500) and the FLAG-tagged mouse mitofilin construct in pcDNA3 plasmid vector were generously provided by Dr. Jiping Zha, formerly of University of Texas Southwestern (John et al, 2005). The mitochondrially-targeted eYFP protein expression construct in pcDNA3 plasmid vector (mitoYFP) and pcDNA3 empty vector plasmid (pcDNA3; Invitrogen) were generously provided by Dr. Don DeFranco, University of Pittsburgh. HuSH 29mer shRNA constructs against human mitofilin were purchased from OriGene Technologies (cat#TR312153; Rockville, MD). The provided kit included an empty vector pRS plasmid (TR20003) negative control, a non-functional GFP-targeted shRNA construct (TR30003) negative control, and four functional human mitofilin targeted shRNA constructs, TI348605 (TI’05), TI348606 (TI’06), TI348607 (TI’07), and TI348608 (TI’08). All plasmids used were initially transduced into competent DH5 α bacteria (Invitrogen), cultured in ampicillin LB media, and purified using QiaGen Midiprep or Miniprep kits (QiaGen, Valencia, CA).

Transient Transfection and Treatment of Differentiated PC12 Cells

PC12 cells were subcultured on rat-tail collagen coated 6-well plates at 1.125×10^5 cells/well and differentiated in DMEM supplemented with 1% HS, 1% FBS, 1% pen/strep, and 100 ng/ml NGF (PC12 differentiation media) for a total of 5-6 days. On day 3 of differentiation, media was removed (conditioned media) and replaced with 2.5 mL OptiMEM supplemented with 0.05 $\mu\text{g/mL}$ NGF, and cells were transfected with 2 μg plasmid DNA and 6 μL Lipofectamine™ 2000 according to manufacturer's instructions. Transfection media was removed after 3.5 hrs and replaced with a 1:1 mixture of conditioned media and fresh PC12 differentiation media for 16-18 hrs, then replaced with full fresh PC12 differentiation media. Non-transfected controls underwent the same media changes, without transfection reagents or DNA present. The described method results in approximately 10-12% transfection rate in differentiated PC12 cells based on co-transfection with mitoYFP (data not shown).

On day 6 of differentiation (72 hr following transient transfection), cells were treated with either DA or rotenone. For DA toxicity experiments, media was replaced with fresh differentiating media with or without 150 μM DA and cells were incubated for 24 hrs, a concentration and time period demonstrated to elicit moderate cell death. For rotenone toxicity experiments, rotenone was diluted in a 1:1 mixture of DMSO and sterile H_2O . Cell media was replaced with fresh PC12 differentiation media with either 0.5 μM rotenone or equivalent volume of vehicle added, as previously described (Dukes et al., 2005). The presence of DMSO did not affect the viability of the cells (data not shown).

Stable plasmid expression and treatment in PC12 cells

Proliferating PC12 cells, maintained in DMEM supplemented with 7% HS, 7% FBS, and 1% pen/strep (PC12 media), were plated at 1×10^6 cells/plate on collagen-coated 6 cm plates. After 48hrs, at ~75% confluency, cells were transfected in 5 mL OptiMEM, using 4 μ g plasmid DNA and 12 μ L Lipofectamine™ 2000. Transfection media was removed after 5hr and replaced with 1:1 conditioned media:fresh PC12 media for 18hr, followed by a media change to full PC12 media. For selection, cells were treated with 500 μ g/mL Geneticin ® (G418) for 5 days, followed by 375 μ g/mL G418 for 5 days, and finally maintained in PC12 media supplemented with 200 μ g/mL G418 to ensure selection of stable cells. Prior to DA exposure, cells were differentiated with NGF for 6 days with G418 present, and then treated with 150 μ M DA for 24 hr. For rotenone treatments, cells were differentiated with NGF for 5 days without G418, and then treated with 0.5 μ M rotenone for 48 hr. Cells were also collected following 5 days of differentiation for Western blot analyses of mitofilin expression.

Transient transfection and treatment of SH-SY5Y cells

Human-derived SH-SY5Y neuroblastoma cells were subcultured on 6-well plates at 2×10^5 cells/well in DMEM supplemented with 10% FBS and 1% pen/strep (SH media). The cell media was changed 48hr after plating and supplemented with 20 μ M retinoic acid (SH differentiation media) and the cells differentiated for a total of 5 days. On day 2 of differentiation, media was removed and replaced with a 1:1 mixture of OptiMEM and SH media lacking penicillin/streptomycin and supplemented with 5-10 μ M retinoic acid. Cells were transfected with 2 μ g plasmid DNA and 6 μ L Lipofectamine™ 2000 according to manufacturer's instructions. Transfection media was removed after 3.5 hrs, and replaced with a 1:1 mixture of

penicillin/streptomycin-free SH media and full SH differentiation media 16-18 hr, and then replaced with full fresh SH differentiation media. Non-transfected controls underwent the same media changes, without transfection reagents or DNA present. Based on imaging of differentiated SH-SY5Y cells co-transfected with mitoYFP, we achieved approximately a 60% transfection rate (data not shown). On day 5 of differentiation (72hr following transient transfection), media was replaced with fresh SH differentiating media with or without 150-250 μ M DA and cells were incubated for 24 hrs.

Stable expression of mitofilin and treatment in SH-SY5Y cells

Proliferating SH-SY5Y cells, maintained in SH media, were plated at 1.5×10^6 cells/plate in 6cm plates. At 24 hrs after plating (~80% confluency) cells were transfected as described above using 4 μ g plasmid DNA and 12 μ L Lipofectamine™ 2000. Stable cells were selected by treating media with 700 μ g/mL G418 for 4 days, followed by 500 μ g/mL for 4 days. Proliferating cells were maintained in SH media supplemented with 250 μ g/mL G418. Cells were differentiated in SH media supplemented with 20 μ M retinoic-acid for 5 days, without G418. On day 5, cells were either collected for Western blot analyses, or treated with media containing 250 μ M DA for 24 hr or 0.5 μ M rotenone for 48 hr, followed by analyzing cell viability. Non-transfected cells were maintained in parallel to the stable cells during selection, without G418, to serve as a passage-matched control.

Cell Collection and Viability Assay

Following treatment, SH-SY5Y cells were collected by 1 min exposure to 500 μ L trypsin followed by force pipetting with SH media, rinsed with PBS, and isolated by centrifugation.

PC12 cells were harvested by force pipetting without trypsin, rinsed, and isolated by centrifugation. To determine changes in cell viability, cells were resuspended in PBS and an aliquot was used for cell counting in the trypan blue exclusion assay. In all cases, cell viability in each group was compared to its respective, untreated or vehicle-treated control to determine percent cell death due to toxin treatment. For Western blot analysis, collected cells were resuspended in lysis buffer (9 M urea, 2% w/v CHAPS, and 30 mM Tris-base, pH 8.0) with protease inhibitor cocktail. Final protein concentrations were determined by the Bradford method (Bradford, 1976).

SDS-PAGE and Western Blot Immunodetection of Select Proteins

Lysed whole-cell protein samples (25-50 µg/lane) were run on 5-20% gradient SDS-PAGE (Hoefer ® Mighty Small II apparatus) and transferred to nitrocellulose (0.2 µm; BioRad) via a BioRad Trans-Blot ® Semi-Dry Electrophoretic Transfer system for Western blot analysis. Blots were blocked with LiCor blocking buffer supplemented with 0.2% w/v fat-free dry milk, and then exposed to primary antibody in blocking buffer with 0.1% Tween-20 16-18 hrs at 4°C. Immunoreactive bands were detected using the LiCor IRDye secondary antibodies as described above. Blots were imaged and quantified using a LiCor Odyssey imaging system coupled to Odyssey analysis software ver. 3.0.

Statistical Analysis

For all experiments, results were analyzed by single-factor ANOVA followed by *pos-hoc* Tukey's T-test, for equal sample sizes between groups, or Fisher's protected T-test, for unequal sample sizes. Significance was determined at $p < 0.05$.

5.4 RESULTS

5.4.1 Transient Overexpression of FLAG-tagged mouse mitofilin protects against DA- and rotenone-induced toxicity in differentiated PC12 cells

In the initial experiments, we sought to examine whether increased mitofilin would affect the cellular response to toxin exposure. NGF-differentiated PC12 cells were transiently transfected with either empty-vector pcDNA3 plasmid (pcDNA3) or FLAG-tagged mouse mitofilin plasmid (Mitofilin-FLAG) as described, while non-transfected control cells (Control) underwent only media changes. Western blot analysis of cells collected 72 hr following transfection detected two bands immunoreactive for mitofilin (at approximately 92 and 82 kDa) in Control and pcDNA3 cells (**Figure 12A**). In the Mitofilin-FLAG cells, Western blot also confirmed the presence of the FLAG-tag associated with a third band immunoreactive for mitofilin at the expected molecular weight for the mitochondrial-processed form of the expressed protein (approximately 87 kDa; Figure 1A) (John et al., 2005). These results confirm plasmid expression and processing of the final protein product. Quantification showed similar levels in endogenous mitofilin between Control and pcDNA3 cells, and significantly increased total mitofilin expression in transiently transfected Mitofilin-FLAG cells (+80.9%, n=5) as compared to Control cells (**Figure 12B**).

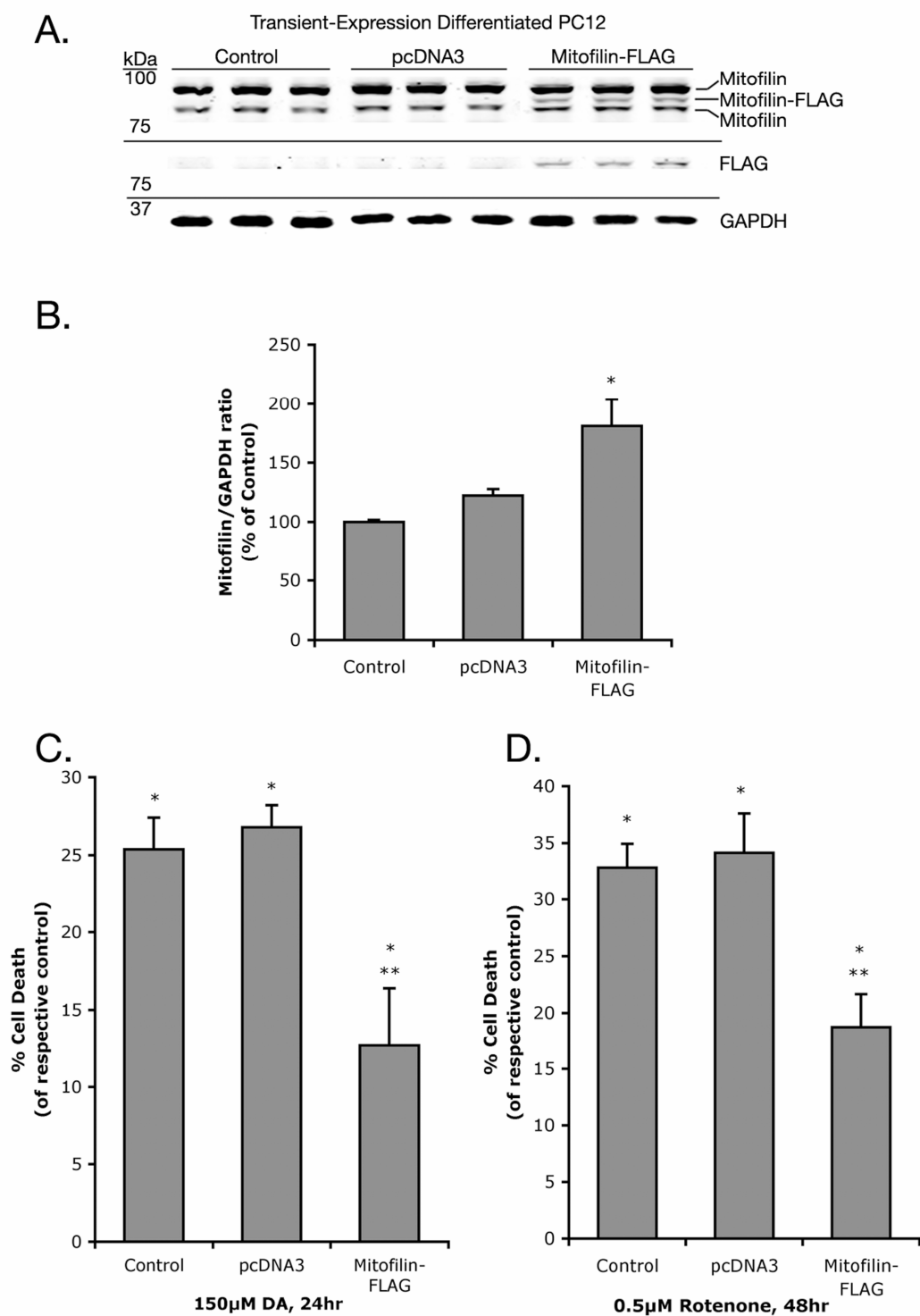


Figure 12: Transient overexpression of FLAG-tagged mitofilin in differentiated PC12 cells.

Figure 12 (continued): Transient overexpression of FLAG-tagged mitofilin in differentiated PC12 cells.

(A) Differentiated PC12 cells were collected 72 hr following transient transfection of empty vector pcDNA3 (pcDNA3) or FLAG-tagged mouse mitofilin (Mitofilin-FLAG), with non-transfected Control cells. Whole-cell lysate was examined by Western blot using T3867 rabbit anti-mitofilin. A band corresponding to FLAG-tagged mitofilin was observed in addition to endogenous forms of mitofilin in mitofilin-FLAG cells. (B) Quantification of mitofilin from Western blots with respect to the GAPDH loading control in differentiated PC12 pcDNA3 and Mitofilin-FLAG cells as compared to non-transfected Control (n=5, mean \pm SEM; * = $p < 0.05$). (C,D) Cell death was assessed by trypan blue exclusion for control and treated groups, and is represented as % cell death as compared to respective untreated controls following (C) 24 hr treatment with 150 μ M DA (n=5) and (D) 48 hr treatment with 0.5 μ M rotenone exposure (n=7; mean \pm SEM; * = significant from untreated control, ** = Mitofilin-FLAG cell death significant from pcDNA3 and Control, $p < 0.05$).

Three days after transfection, differentiated cells were treated with either 150 μ M DA or control media for 24 hrs (n=5), or with 0.5 μ M rotenone or vehicle for 48hrs (n=7). Transient expression of the pcDNA3 empty vector or Mitofilin-FLAG did not significantly alter cell viability in control-media treated groups as compared to non-transfected Control. Significant cell death was elicited by DA or rotenone exposure in both non-transfected Control cells (25.4% cell death with DA; 32.8% cell death with rotenone) and empty vector pcDNA3 cells (26.8% cell death with DA; 34.1% cell death with rotenone) as compared to their respective non-treated control cells (**Figure 12C,D**). In Mitofilin-FLAG cells, DA-induced cell death was significantly attenuated by 50%, and rotenone-induced cell death significantly attenuated by 43% as compared to treated Control and pcDNA3 cells (**Figure 12C,D**).

5.4.2 Transient Overexpression of FLAG-tagged mouse mitofilin protects against DA-induced toxicity in differentiated SH-SY5Y cells

SH-SY5Y cells were differentiated for five days with retinoic acid (RA, 20 μ M), and were transiently transfected with either pcDNA3 or Mitofilin-FLAG vectors on day two of differentiation. Western blot analysis of cells collected 72 hr after transfection detected one immunoreactive band for mitofilin, or a tightly-associated doublet, at the appropriate molecular weight (approximately 87 kDa; **Figure 13A**). Quantification of mitofilin bands showed no significant difference in endogenous mitofilin levels between Control and pcDNA3-transfected cells, and confirmed overexpression of mitofilin in Mitofilin-FLAG cells (+117.9% above Control cells, n=4). This band was also associated with the presence of the FLAG-tag in the Mitofilin-FLAG cells (**Figure 13A,B**).

Three days following transfection, cells were treated with 150 μ M DA (n=6), 250 μ M DA (n=3), or untreated control media for 24 hrs. DA exposure resulted in significant cell death in both the empty vector pcDNA3 cells (11.4% with 150 μ M DA; 27.0% with 250 μ M DA) and non-transfected Control cells (7.4% with 150 μ M DA; 19.9% with 250 μ M DA; **Figure 13C,D**). DA-induced cell death was significantly attenuated in the Mitofilin-FLAG cells by 47-65% with 150 μ M DA, and by 45-59% following 250 μ M DA as compared to DA-treated empty vector and non-transfection control groups.

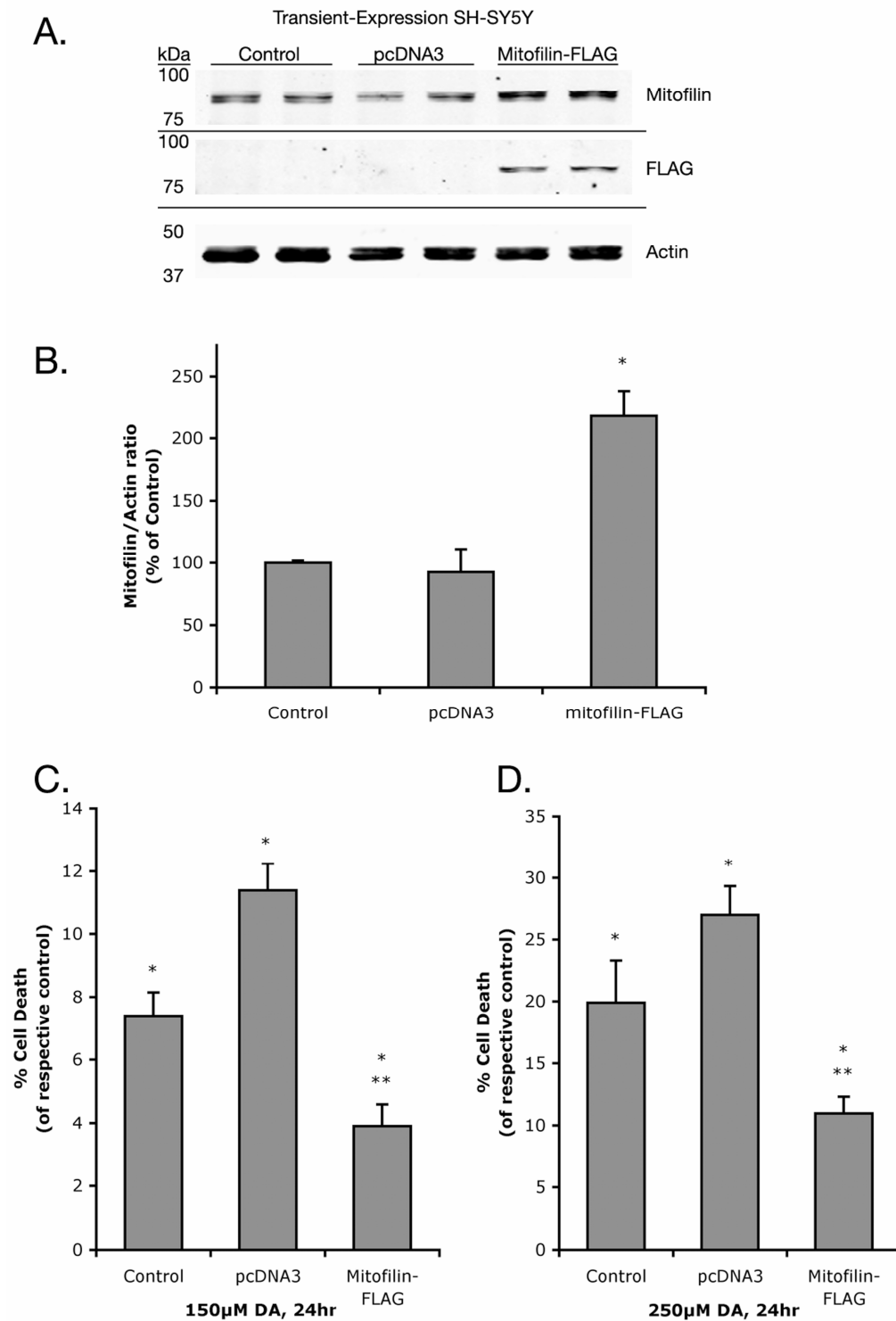


Figure 13: Transient overexpression of FLAG-tagged mitofilin in differentiated SH-SY5Y cells.

Figure 13 (continued): Transient overexpression of FLAG-tagged mitofilin in differentiated SH-SY5Y cells.

(A) Differentiated SH-SY5Y cells were collected 72 hr following transient transfection of empty vector pcDNA3 (pcDNA3) or FLAG-tagged mouse mitofilin (Mitofilin-FLAG), with non-transfected Control cells. Whole-cell lysate was examined by Western blot using Genemed rabbit anti-mitofilin. A FLAG-immunoreactive band was detected corresponding to the same molecular weight as mitofilin. (B) Quantification of mitofilin from Western blots with respect to the actin loading control in differentiated SH-SY5Y cells as compared to non-transfected Control (n=4, mean \pm SEM; * p<0.05). (C,D) Viability was assessed by trypan blue exclusion for control and treated groups, and is represented as % cell death as compared to respective untreated controls following (C) 24 hr treatment with 150 μ M DA (n=6) and (D) 24 hr treatment with 250 μ M DA exposure (n=3; mean \pm SEM; * = significant from untreated control, ** = Mitofilin-FLAG cell death significant from pcDNA3 and Control, p<0.05).

5.4.3 Stable overexpression of FLAG-tagged mitofilin is protective against both DA and rotenone toxicity in differentiated PC12 and SH-SY5Y cells

Proliferating PC12 and SH-SY5Y cells were stably transfected with either empty-vector pcDNA3, mitochondria-targeted fluorescent YFP expression vector (mitoYFP, to control for expressing a mitochondria-targeted protein), or Mitofilin-FLAG. Stable cells were selected with and maintained in media containing Geneticin® (G418) as described in Methods. Western blot analyses of NGF-differentiated stable-expressing PC12 cells (**Figure 14A,B**) and RA-differentiated stable-expressing SH-SY5Y cells (**Figure 15A,B**) showed there was no difference in endogenous mitofilin between pcDNA3- and mitoYFP-stable cells within cell types. However, Mitofilin expression was significantly increased above stable-pcDNA3 cells in both

stable-Mitofilin-FLAG cell lines (+40.3% in PC12 cells, n=6; +107.3% in SH-SY5Y cells, n=4), and was associated with detection of the FLAG-tag (**Figures 14 and 15**).

Differentiated stable PC12 cell lines were treated with 150 μ M DA for 24 hr (n=8), 0.5 μ M rotenone for 48 hr (n=6), or non-treated media, as described in Methods. Both DA and rotenone exposure elicited significant cell death in differentiated mitoYFP stable cells (22.1% with DA; 26.3% with rotenone) and pcDNA3 stable cells (24.9% with DA; 28.7% with rotenone) compared to their untreated controls (**Figure 14C,D**). By comparison, differentiated stable Mitofilin-FLAG PC12 cells showed approximately 50% less cell death following either DA or rotenone treatment (**Figure 14C,D**). There was no difference in viability between untreated stably-transfected cell lines.

Significant cell death was also observed following 250 μ M DA treatment (n=6) or 0.5 μ M rotenone treatment (n=6) in differentiated stable SH-SY5Y mitoYFP cells (43.9% with DA; 14.0% with rotenone), pcDNA3 cells (45.5% with DA; 11.9% with rotenone), and passage-matched non-transfected Control cells (49.7% with DA; 12.6 % with rotenone). Treated Mitofilin-FLAG stable SH-SY5Y cells demonstrated significantly lower cell death (32.1% cell death with DA, ~30% attenuated; 4.2% cell death with rotenone, ~65% attenuated) compared to treated controls (**Figure 15C,D**). In SH-SY5Y stable cell lines and non-transfected Control cells, there was no difference in viability between untreated cells. Results from both the transient-transfected and stable-transfected cells suggest that increased mitofilin expression decreases the vulnerability of dopaminergic cells to the toxins DA and rotenone.

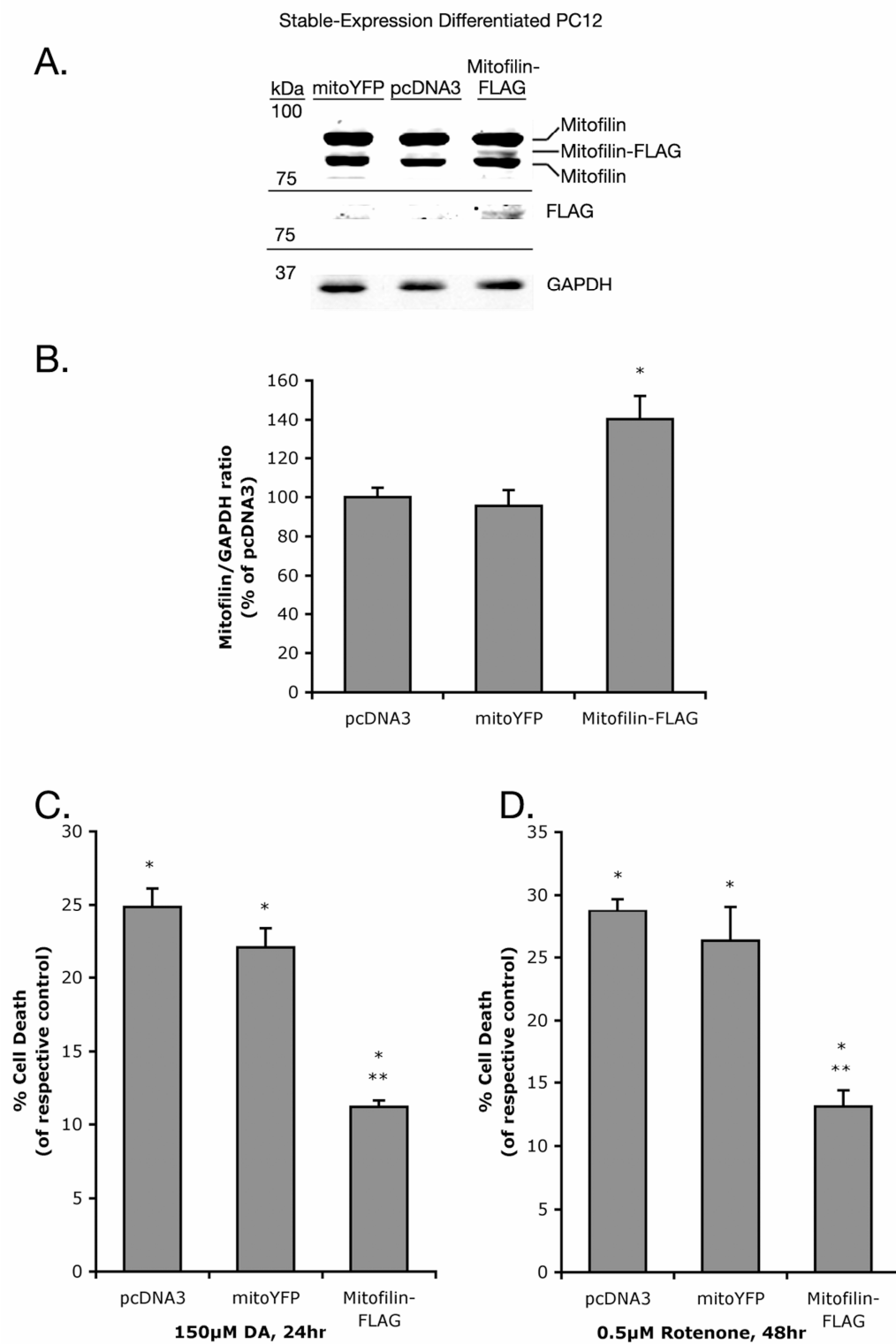


Figure 14: Stable expression of FLAG-tagged mitofilin in differentiated PC12 cells.

Figure 14 (continued): Stable expression of FLAG-tagged mitofilin in differentiated PC12 cells.

(A) Stable expressing Mitofilin-FLAG, pcDNA3 empty vector, and mitoYFP PC12 cells were differentiated for 5 days, collected, and whole-cell lysate examined by Western blot using T3867 rabbit anti-mitofilin. A mitofilin- and FLAG-immunoreactive band corresponding to FLAG-tagged mitofilin was observed in mitofilin-FLAG cells in addition to endogenous forms of mitofilin. (B) Quantification of mitofilin from Western blots with respect to the GAPDH loading control in differentiated stable Mitofilin-FLAG and mitoYFP PC12 cells as compared to stable pcDNA3 cells (n=6, mean \pm SEM; * p<0.05). (C,D) Viability was assessed by trypan blue exclusion for control and treated groups, and is represented as % cell death from respective untreated controls following (C) 24 hr treatment with 150 μ M DA exposure (n=8), treated following 6 days of differentiation, and (D) 48 hr treatment with 0.5 μ M rotenone exposure (n=6), treated following 5 days of differentiation. (mean \pm SEM; * = significant from untreated control, ** = Mitofilin-FLAG cell death significant from pcDNA3 and mitoYFP, p<0.05).

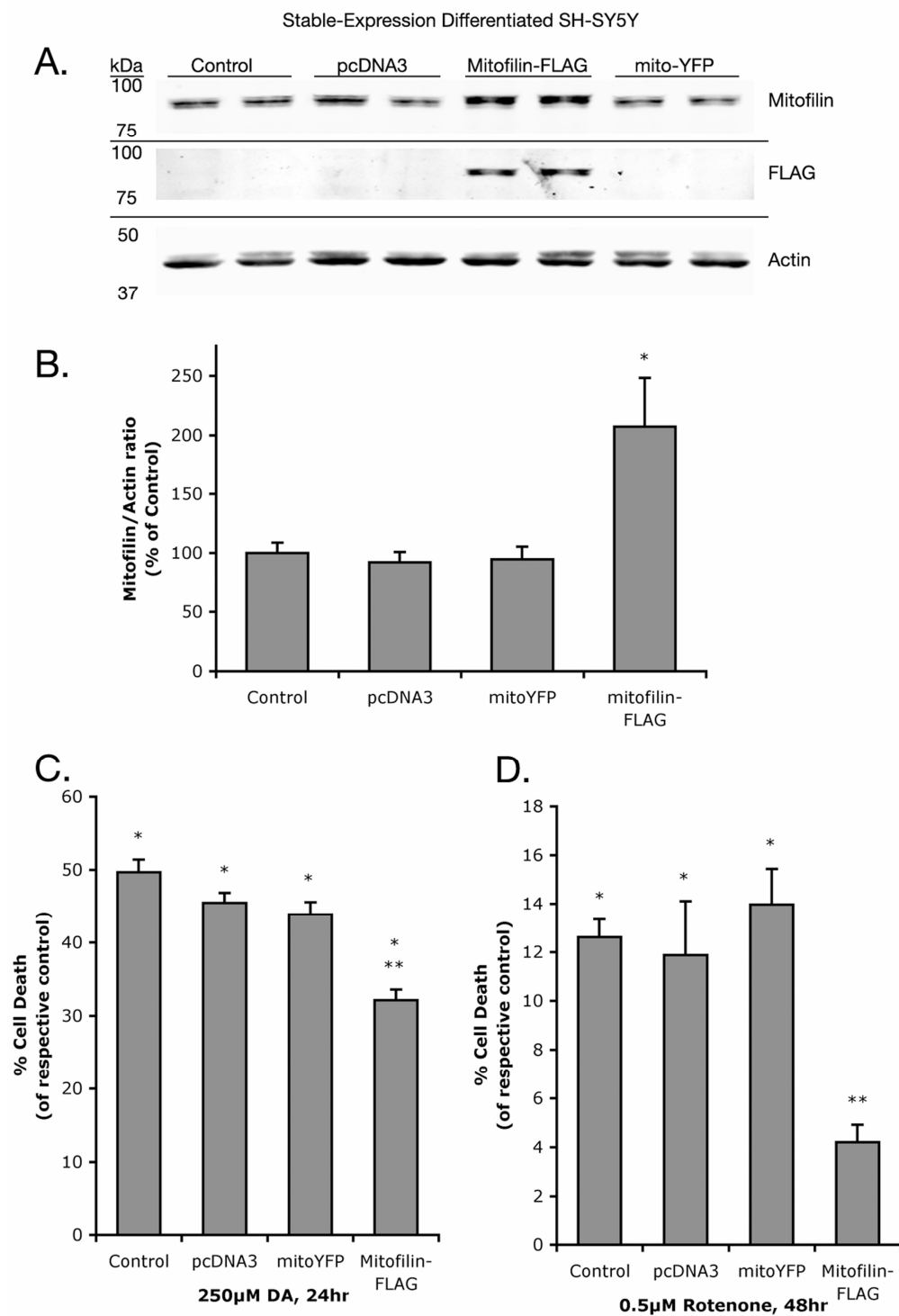


Figure 15: Stable expression of FLAG-tagged mitofilin in differentiated SH-SY5Y cells.

Figure 15 (continued): Stable expression of FLAG-tagged mitofilin in differentiated SH-SY5Y cells.

(A) Non-transfected, passage-matched Control SH-SY5Y cells and stable-expressing Mitofilin-FLAG, pcDNA3 empty vector, and mitoYFP SH-SY5Y cells were differentiated for 5 days, collected, and whole-cell lysate examined by Western blot using T3867 rabbit anti-mitofilin. A FLAG-immunoreactive band was detected corresponding to the same molecular weight as mitofilin. (B) Quantification of mitofilin from Western blots with respect to the actin loading control in differentiated SH-SY5Y Mitofilin-FLAG, pcDNA3, and mitoYFP cells as compared to non-transfected control cells (n=4, mean \pm SEM; * p<0.05). (C,D) Viability was assessed by trypan blue exclusion for control and treated groups, and is represented as % cell death from respective untreated controls following (C) 24 hr treatment with 250 μ M DA exposure (n=6) and (D) 48 hr treatment with 0.5 μ M rotenone exposure (n=6; mean \pm SEM; * = significant from untreated control, ** = Mitofilin-FLAG cell death significant from pcDNA3, mitoYFP, and Control cells, p<0.05).

5.4.4 Loss of mitofilin increases susceptibility of differentiated SH-SY5Y cells to DA-induced toxicity

Differentiated SH-SY5Y cells were transiently transfected as described with one of five shRNA vectors in pRS plasmid: empty vector (TR20003, n=5), non-functional GFP shRNA vector (TR30003, n=3), and three functional shRNA vectors against human mitofilin (TI'05, TI'07, and TI'08; n=4). Western blot analysis of cells collected 3 d following transfection confirmed a significant knock down of total mitofilin protein by 23-30% as compared to the TR20003 empty-vector control (**Figure 16A,B**). Transfected cells and non-transfected controls

were treated 3 d after transfection with media containing 250 μ M DA or control media for 24 hrs. Again, transfection did not have a significant effect on viability in non-treated cells. DA exposure of SH-SY5Y cells resulted in 13% percent cell death in Control cells and 17-18% cell death in transfection-control TR20003 and TR30003 cells (n=6; **Figure 16C**), comparable to that seen in DA-treated Control SH-SY5Y cells as discussed above. DA-induced cell death was potentiated by 33-36% in cells transfected with TI'05, TI'07, and TI'08 functional-shRNAs, which decreased endogenous mitofilin (n=6; **Figure 16**).

One functional shRNA-mitofilin, TI'05, contained a 29-mer sequence not compatible with known mouse mitofilin mRNA sequences. To examine whether the increased vulnerability to DA-induced toxicity observed with shRNA expression could be rescued by mitofilin, differentiated SH-SY5Y cells were co-transfected with one of three combinations of plasmid DNA: (1) transfection-control, TR30003 non-functional shRNA and pcDNA3 empty vector (TR30003+pcDNA3); (2) knockdown-control, pcDNA3 and TI'05 shRNA (TI'05+pcDNA3); or (3) knockdown and rescue; TI'05 shRNA and mouse Mitofilin-FLAG (TI'05+Mitofilin-FLAG). Percent cell death following treatment with 250 μ M DA was significantly increased in the knockdown TI'05+pcDNA3 cells (35.2%, n=3) as compared to the transfection-control TR30003+pcDNA3 cells (19.0%, n=3; **Figure 17**). Co-transfection of functional shRNA TI'05 with Mitofilin-FLAG eliminated the TI'05-mediated potentiation of DA toxicity, reducing cell death in the knockdown and rescue TI'05+Mitofilin-FLAG cells to 20.3% of untreated control (n=4). Cell death in this group was not significantly different from the DA-treated transfection control (**Figure 17**). There was also no significant difference in viability among non-DA-treated groups. Together, the above data suggest that a loss of mitofilin increases the vulnerability of dopaminergic cells to DA-induced toxicity.

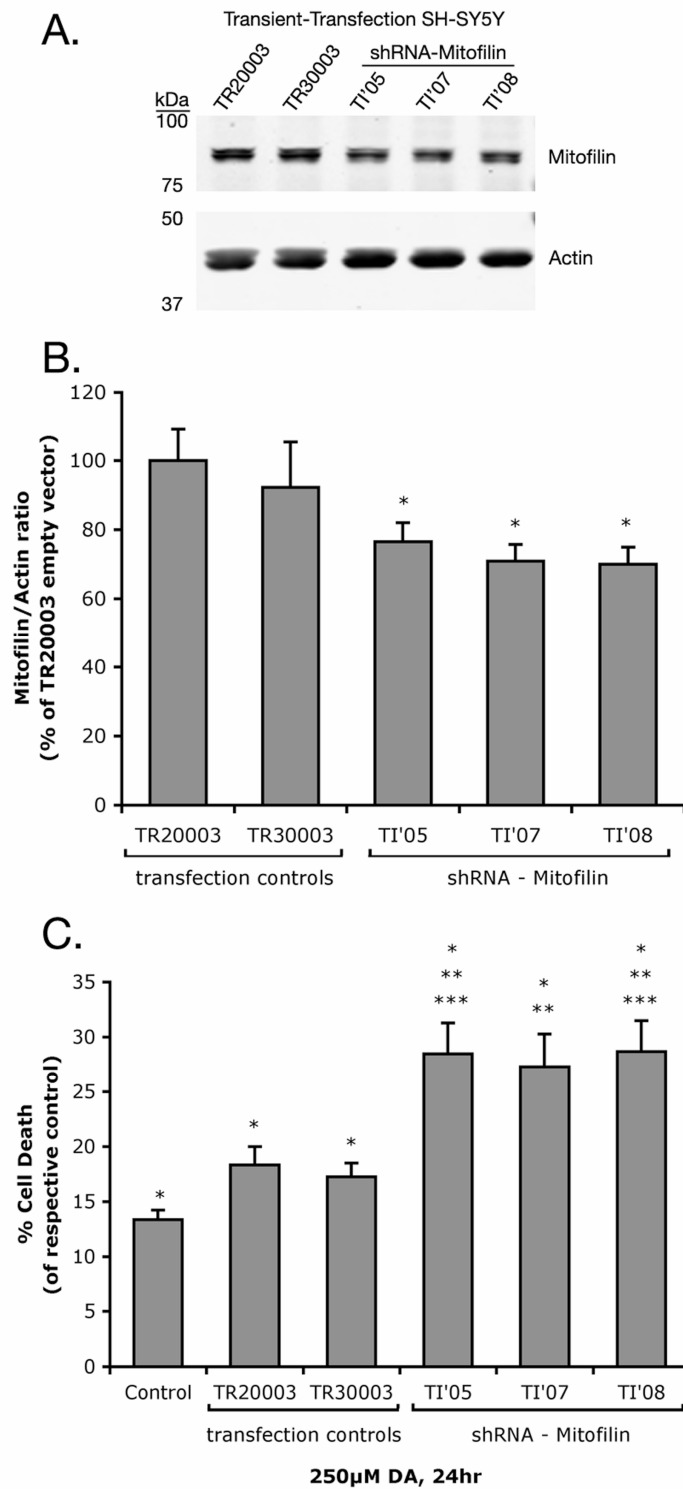


Figure 16: Transient expression of shRNA-mitofilin and knockdown of mitofilin in differentiated SH-SY5Y cells.

Figure 16 (continued): Transient expression of shRNA-mitofilin and knockdown of mitofilin in differentiated SH-SY5Y cells.

(A) Differentiating SH-SY5Y cells were transfected with empty-vector pRS plasmid (TR20003), non-functional shRNA-GFP vector (TR30003), or one of three functional shRNA-mitofilin vectors (TI'05, TI'06, TI'08). Differentiated cells were collected 72 hr following transient transfection and whole-cell lysate examined by Western blot using Genemed rabbit anti-mitofilin. (B) Quantification of Western blots with respect to the actin loading control in shRNA-mitofilin cells (n=4), and TR30003 cells (n=3) as compared to TR20003 transfection control cells (n=5; mean \pm SEM; * $p < 0.05$). (C) Viability was assessed by trypan blue exclusion for control and treated groups, and is represented as % cell death from respective untreated controls following 24 hr treatment with 250 μ M DA (n=6, mean \pm SEM, * = significant from untreated control, $p < 0.05$). Cell death was significantly increased in transiently-expressing shRNA-mitofilin cells as compared to treated TR30003 negative control and non-transfected Control (** = $p < 0.05$) and to TR20003 empty vector control (***) = $p < 0.05$).

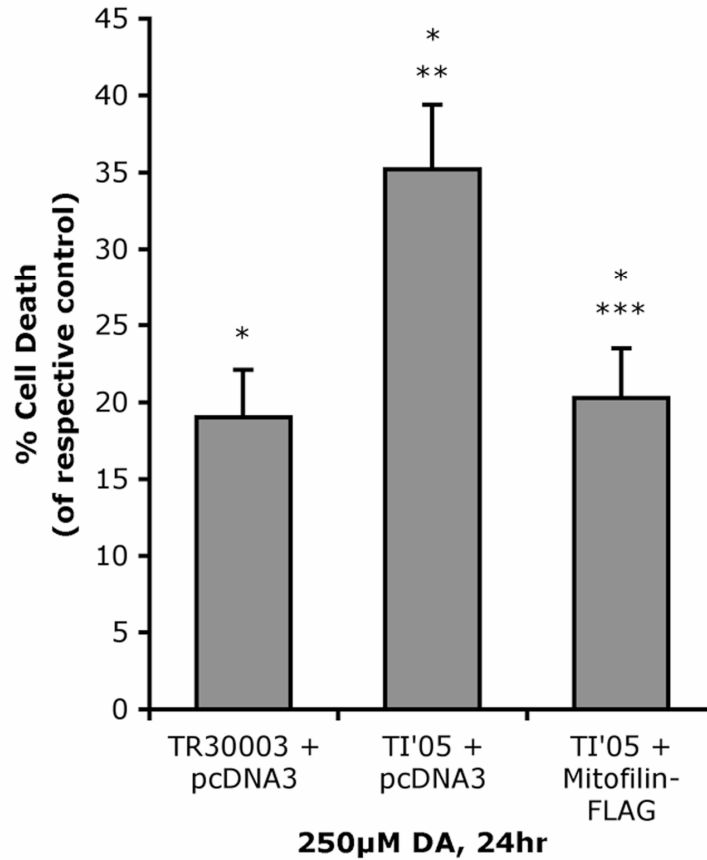


Figure 17: Rescue of SH-SY5Y cells from shRNA-associated effect on DA susceptibility

Differentiated SH-SY5Y cells were co-transfected with one of three combinations: transfection control, TR30003 plus pcDNA3 empty vector (TR30003+pcDNA3, n=3); knockdown control, pcDNA3 empty vector plus shRNA-human mitofilin TI'05 (TI'05+pcDNA3, n=3); and knockdown and rescue, shRNA-mitofilin TI'05 plus mouse-Mitofilin-FLAG vector (TI'05+Mitofilin-FLAG, n=4). Viability was assessed by trypan blue exclusion for control and treated groups, and is represented as % cell death from their respective non-toxin-treated controls following 24 hr treatment with 250 µM DA (n=3-4, mean \pm SEM, *= significant from untreated control, $p < 0.05$). Cell death was significantly increased in DA-treated TI'05+pcDNA3 cells as compared to DA-treated TR30003+pcDNA3 cells (** = $p < 0.05$), while cell death was attenuated in DA-treated TI'05+Mitofilin-FLAG cells as compared to DA-treated TI'05+pcDNA3 cells (*** = $p < 0.05$).

5.5 DISCUSSION

Mitofilin structure and expression has been studied and characterized by several laboratories (Gieffers et al., 1997; Ichio et al., 1994; John et al., 2005; Odgren et al., 1996). Interest in the protein has increased recently following a study by John and colleagues, which described mitofilin as a critical protein for maintaining mitochondrial cristae structure (John et al., 2005). Mitofilin protein levels also appear to be sensitive to oxidative stress (Magi et al., 2004; Van Laar et al., 2008; see **Chapter 3**). We previously demonstrated that mitofilin abundance was decreased 65% in isolated rat brain mitochondria following exposure to DAQ, and significantly decreased in mitochondria isolated from DA-exposed PC12 cells (Van Laar et al., 2008; see **Chapter 3**). In this study, we have demonstrated that modulation of the level of mitofilin affects the vulnerability of dopaminergic cells to two separate toxins, DA and rotenone. To our knowledge, this is the first demonstration of altered mitofilin expression influencing the cellular response to toxic insults. While the mechanism remains elusive, these findings carry implications for the involvement of mitochondrial stability in disease models and pathologies, particularly PD.

5.5.1 Mitofilin overexpression decreases dopaminergic cell vulnerability to DA- and rotenone-induced toxicity

In this study, we found that increased mitofilin could attenuate cell death in response to mitochondrial stressors. Transient overexpression of FLAG-tagged mitofilin significantly decreased cell loss following exogenous DA exposure in both differentiated PC12 and SH-SY5Y cells, as well as against rotenone exposure in PC12 cells. We also generated stable cell lines

overexpressing mitofilin. Again, we observed attenuated cell death in stable mitofilin-overexpressing PC12 and SH-SY5Y cells exposed to both DA and rotenone as compared to stable-expressing empty vector and mitoYFP cells. These findings suggest that excess mitofilin may increase cellular tolerance against stressors, such as the PD model toxins DA and rotenone.

Both the DA and rotenone toxicity models have been well established in PC12 and SH-SY5Y cells, both in differentiated and undifferentiated cells, and have been attributed to factors including oxidative stress, protein modification, alterations in mitochondrial respiration and membrane potential, and mitochondrial release of apoptosis-initiating factors (Ben-Shachar et al., 2004; Berman and Hastings, 1999; Brenner-Lavie et al., 2008; Dukes et al., 2005; Imamura et al., 2006; Jones et al., 2000; Lai and Yu, 1997a; Lai and Yu, 1997b; Marella et al., 2007; Molina-Jimenez et al., 2003; Offen et al., 1996; Si et al., 1998; Wang et al., 2005; Watabe and Nakaki, 2007a). Chronic, low-dose rotenone exposure in SH-SY5Y cells also caused decreased mitochondrial membrane potential, as well as altered Ca^{2+} signaling (Sherer et al., 2001). One study found that coenzyme-Q10 supplementation reduced the effect of rotenone toxicity in SH-SY5Y cells by attenuating rotenone-induced loss of the mitochondrial membrane potential (Menke et al., 2003). Mitofilin, through its proposed role in mitochondrial cristae maintenance, may attenuate DA and rotenone toxicity in a similar manner by retaining mitochondrial structural integrity during cellular stress. Further work is necessary to elucidate the protective mechanism of mitofilin.

5.5.2 A loss of mitofilin increases susceptibility of differentiated SH-SY5Y cells to DA toxicity

Mitofilin expression is suggested to be important for cell survival as loss of mitofilin led to a reduced mitotic growth rate in HeLa cells, along with a slight increase in apoptosis (John et al., 2005). Because DA exposure can decrease levels of mitofilin protein in dopaminergic cell mitochondria (Van Laar et al., 2008), we sought to determine whether decreased levels of mitofilin would affect the susceptibility of dopaminergic cells to exogenous DA. At the time point examined in differentiated SH-SY5Y cells, we did not observe an effect on basal viability following mitofilin knockdown via transient expression of shRNA vectors against human mitofilin in SH-SY5Y cells in our experiments. The level of knockdown (~30%) observed in these experiments may not be dramatic enough to affect cell viability without additional stressors. Further work will be necessary to see if modulating mitofilin levels affects basal survivability of dopaminergic cells over time. However, we did find that a shRNA-induced reduction of mitofilin protein in differentiated SH-SY5Y cells was associated with a significant decrease in viability following DA exposure as compared to DA-exposed controls. Further, SH-SY5Y cells were rescued from the shRNA-associated effect by transiently co-expressing human mitofilin shRNA with mouse mitofilin resistant to the RNAi. These results show that decreased mitofilin levels increase the vulnerability of dopaminergic cells to toxic insults.

5.5.3 Mitofilin has been connected to neuropsychiatric disorders and neurodegenerative disease

Alterations in mitofilin expression level and structure have previously been linked to neuropsychiatric and neurodegenerative diseases. Proteomic analysis of brain tissue from fetal Down syndrome subjects has found dysregulated expression of proteins, including mitofilin, which is decreased by nearly 50% from control (Myung et al., 2003). Models have also linked mitofilin levels to neurological disorders. Proteomic analysis of mitochondria from G93A-SOD1-expressing NSC34 cells, a cell line model of familial ALS, found multiple proteins with altered expression, including significantly decreased mitofilin expression (-72%), compared to wild type cells (Fukada et al., 2004). We have shown that exposure of brain mitochondria to an oxidant stressor leads to a 65% decrease in mitofilin protein in an *in vitro* model of PD (Van Laar et al., 2008; see **Chapter 3**)

Alterations in mitofilin structure have also been tied to neurological disorders. Proteomic analysis of hippocampal tissue from senescence-accelerated mouse prone 8 (SAMP8) mice, a model for age-related cognitive deficits and neuronal degeneration, revealed a basic pI shift specifically in mitofilin protein as compared to age-matched SAM resistance control mice (Wang et al., 2008a). Mitofilin was one of only two proteins found to be altered between the two mouse lines, though the specific reason for the pI shift and the impact of any mitofilin protein modification on cellular function are uncertain (Wang et al., 2008a). A basic pI shift was also observed in mitofilin from cortical tissue of a seizure-sensitive strain of Mongolian gerbil (Omori et al., 2002). The authors suggest that sequence changes or alternative splicing of genes may be a factor in generating the different protein isoforms identified (Omori et al., 2002). Other studies have also demonstrated the potential of mitofilin to be post-translationally

modified, which could affect protein pI, including oxidation of tryptophan residues in cardiac muscle mitofilin (Taylor et al., 2003), oxidation of cysteine residues following alcohol exposure in human hepatoma cells (Suh et al., 2004), and poly-ADP-ribosylation of mitofilin in a rat model of traumatic brain injury (Lai et al., 2008). We have also demonstrated covalent modification of mitofilin by DAQ (See **Chapter 4**). Thus, protein structure and modification of mitofilin, in addition to expression level, may be crucial for proper cellular functions.

5.5.4 Conclusions

We have now shown that alterations in mitofilin protein levels modulate vulnerability of two dopaminergic cell lines to two toxin models of PD, DA and rotenone. Though the mechanism remains elusive, recent reports have identified protein-protein interactions of mitofilin that may shed light on a role for mitofilin. Xie et al. found that immunoprecipitation of mitofilin reveals an association with a protein complex including SAM50, coiled-coil-helix coiled-coil-helix domain-containing (CHCHd) proteins 3 and 6, and metaxins 1 and 2, proteins known to be involved in mitochondrial protein import and assembly (Xie et al., 2007). This suggests an integral role for mitofilin in mitochondrial functional maintenance. If mitofilin levels were altered or the protein covalently modified by a stressor, such as oxidized dopamine, the modification could result in dissociation of the protein import complex and reorganization of mitochondrial cristae. Such an event would have a major impact on mitochondrial respiration, protein stability, and ultimately cellular health. Mounting evidence provides a strong case and need for further study to identify the specific roles mitofilin plays in the mitochondria. Understanding mitofilin could lead to a better knowledge of mitochondria-mediated cell death and degeneration pathways, particularly in neurodegenerative disease.

5.6 ACKNOWLEDGMENTS

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6.0 CONCLUDING REMARKS

The pathogenesis of sporadic Parkinson's disease (PD) is thought to result from a combination of environmental and predisposing genetic factors (Warner and Schapira, 2003). Studies also suggest that mitochondrial dysfunction and oxidative stress play key roles in disease pathogenesis. Dopaminergic neurons of the SN, the loss of which is a hallmark of PD, may be particularly vulnerable to degeneration due to DA oxidation. There is evidence of DA oxidation occurring natively in the pigmented neurons of the substantia nigra (SN) (Fornstedt et al., 1986; Zecca et al., 2003). Additionally, conditions that foster disrupted biogenesis of DA or storage of DA could lead to increased levels of cytosolic DA and DA oxidation, increasing neuronal vulnerability (reviewed in Caudle et al., 2008). Using proteomic approaches, I have identified mitochondrial and cellular targets of DA induced modifications. I have also characterized the effects of modulating expression of one of the protein targets, mitofilin, in cell culture models of PD. The proteins targets reported here may have relevance in PD pathogenesis, and should be evaluated for potential therapeutic interventions in PD.

6.1 ISOLATED BRAIN MITOCHONDRIA AND DA OXIDATION AS A MODEL OF PD

In the studies presented in **Chapters 3** and **4** we utilized a model of exposing isolated rat brain mitochondria to oxidized DA, DA quinone (DAQ). Various studies from multiple laboratories, including the Hastings laboratory, have demonstrated that exposure of intact brain mitochondria to DA and DA oxidation products resulted in altered respiration (Berman and Hastings, 1999; Gluck et al., 2002; Gluck and Zeevalk, 2004). Results suggested that DA was modifying the activity of mitochondrial proteins, likely through direct conjugation, but it was unknown which proteins were susceptible to DA modification. Some studies examined the effect of DA on the activities of specific electron transport chain (ETC) complexes to elucidate the targets of DA modification. However, these experiments were carried out in disrupted mitochondria (Ben-Shachar et al., 2004; Khan et al., 2005; Morikawa et al., 1996; Przedborski et al., 1993). No study prior to those comprising this thesis examined which specific mitochondrial proteins are direct targets of DA-induced modification in intact brain mitochondria. Doing such experiments in intact mitochondria is more physiologically relevant, as certain proteins may be inaccessible to DA due to native protein-protein interactions, location, or potentially by the limited ability of DA to cross membranes. Additionally, direct effects of DA oxidation on mitochondrial proteins may involve alterations in multiple interacting proteins. Mitochondrial disruption may destroy such networks, potentially altering the observed effects of DA oxidation.

Notably, the studies in this thesis demonstrated that the electrophilic DAQ could elicit effects on protein abundance and covalently modify proteins throughout the mitochondria, including proteins of the intermembrane space and the matrix. As we observe covalent modification of matrix proteins, such as HSP60 and aconitase, the DAQ must first be gaining

access to this region of the mitochondria. Recent studies in the Hastings and Greenamyre laboratories show that exposure of intact mitochondria to DAQ resulted in covalent modification of Complex I subunits and a significant decrease in Complex I activity (Arduini et al., Society for Neuroscience Abstract 2008; unpublished data). These results suggest that DAQ can access the interior of intact mitochondria, though the mechanism is unknown. A recent study suggested that intact mitochondria could accumulate DA *in vitro* (Brenner-Lavie et al., 2008). Brenner-Lavie et al. found that intact isolated rat brain mitochondria in the presence of a low DA concentration (2 nM) rapidly accumulated (Brenner-Lavie et al., 2008). The accumulation appeared to be an energy- and ion-dependent process, suggestive of a transporter-mediated process, though the specific mechanism is not known (Brenner-Lavie et al., 2008), nor is it known if a similar mechanism works for the DAQ radical. Nevertheless, the ability of DA and DAQ to enter the mitochondria suggests that cytosolic DA could impact mitochondrial function by oxidatively modifying critical mitochondrial proteins.

In support of this hypothesis, other models of PD and dopaminergic cell death suggest that dysregulation of cytosolic DA is relevant to mitochondrial dysfunction and PD pathology. Studies that utilized disruption of the DA storage system by altering the abundance or function of the vesicular monoamine transporter (VMAT2) demonstrated the importance of proper cellular handling and storage of DA (reviewed in Caudle et al., 2008). A recent study reported that mice expressing only 5% of normal levels VMAT2 displayed age-dependant decreases in SN antioxidants, increases in SN cysteinyl-catechol adducts, oxidative damage to proteins, decreased striatal DA, increased alpha-synuclein deposition in SN, and progressive loss of SN dopaminergic neurons when compared to wild types (Caudle et al., 2007). These results

replicate multiple characteristics of nigrostriatal degeneration in PD, and suggest that a deficient system for DA handling may contribute to dopaminergic vulnerability.

Models that utilize inhibitors of mitochondrial respiration also support the hypothesis that DA may contribute to dopaminergic neuron vulnerability. Chronic administration of the Complex I inhibitor rotenone *in vivo* in rats, despite being systemic, results in relatively selective degeneration of nigrostriatal dopaminergic neurons (Betarbet et al., 2000; Sherer et al., 2003a; Sherer et al., 2003b), suggesting an inherent vulnerability of these neurons that may be linked to DA. Cell culture models using mitochondrial toxins have sought to elucidate the role of endogenous and/or excess cytosolic DA in dopaminergic neuron vulnerability. In examining the role of endogenous stores of DA in acute rotenone-induced toxicity in dopaminergic SH-SY5Y human neuroblastoma cells, Watabe and Nakaki found that decreasing or increasing cellular content of endogenous DA attenuated or potentiated rotenone-induced apoptosis, respectively (Watabe and Nakaki, 2007b). In addition, the study found that rotenone exposure led to a redistribution of DA from vesicles to the cytosol (Watabe and Nakaki, 2007b). Additionally, our lab previously demonstrated that co-treatment of differentiated PC12 cells with rotenone and methamphetamine, which induces DA release from vesicles, led to an increase in toxicity compared to rotenone treatment alone (Dukes et al., 2005). The enhanced toxicity observed with co-treatment was eliminated by prior depletion of cellular DA (Dukes et al., 2005), demonstrating that excess cytosolic DA can exacerbate the effect of rotenone. Similarly, Lee et al. found that co-administration of DA and the Complex I inhibitor MPP⁺ increased toxicity over either toxin alone in PC12 cells (Lee et al., 2003). Together, these findings suggest that DA plays a role in the vulnerability of dopaminergic neurons to mitochondrial toxins, and that acute mitochondrial dysfunction may play a role in DA dysregulation.

Cell culture studies also suggest that excess cytosolic DA may have a direct effect on mitochondrial function. Multiple studies have found that DA exposure in neuroblastoma cells resulted in decreased mitochondrial membrane potential in association with cell death (Brenner-Lavie et al., 2008; Fuentes et al., 2007; Gimenez-Xavier et al., 2006; Premkumar and Simantov, 2002; Zafar et al., 2006). Treatment with exogenous DA, in the presence of the MAO inhibitor pargyline, decreased ATP levels in SH-SY5Y cells (Ben-Shachar et al., 2004) suggesting altered ETC function was associated with DA exposure. Premkumar and Simantov found that DA toxicity in human neuronal NMB cells decreased mitochondrial VDAC expression, in addition to dissipating mitochondrial membrane potential (Premkumar and Simantov, 2002). Overexpression of VDAC, a protein integral in formation of the permeability transition pore (PTP) and that we observed to be modified by DAQ (see **Chapters 3 and 4**), was able to protect against DA toxicity (Premkumar and Simantov, 2002). In SK-N-MC cells, DA-induced loss of mitochondrial membrane potential and cell death were abrogated by NAD(P)H quinone oxidoreductase 1 (NQO1) overexpression (Zafar et al., 2006). Superoxide dismutase (SOD) and catalase added to the cell media were not able to prevent DA-associated death (Zafar et al., 2006). As intracellular NQO1 was protective, these findings support the hypothesis that intracellular oxidation of DA is responsible for cell death associated with DA toxicity (Zafar et al., 2006). In RCSN-3 cells, derived from rat SN, VMAT2 inhibition by reserpine in addition to exogenous DA exposure resulted in blebbing of cellular processes, nuclear chromatin condensation, cell death, and mitochondrial damage in the form of structural abnormalities (Fuentes et al., 2007).

These studies suggest a link between DA mishandling, excess cytosolic DA, and mitochondrial dysfunction. Such an effect could ultimately set up a vicious cycle, in which

increased cytosolic DA will oxidize to DAQ in the cytosol and/or accumulate in the mitochondria. DAQ entering the mitochondria would result in modification of mitochondrial proteins and their functions, leading to further increases in DA mishandling, ROS production, and oxidative damage. Such a cycle would likely enhance the vulnerability of dopaminergic neurons following exposure to a stressor.

6.2 PROTEOMICS AS A TOOL FOR DA OXIDATION MODEL RESEARCH

The use of proteomics in neuroscience and the study of neurodegenerative disease has become widely prevalent (Butterfield et al., 2006; Fountoulakis and Kossida, 2006; Gillardon, 2006; Johnson et al., 2005; Kim et al., 2004), and been proposed as the optimal route to characterize diseases such as PD (Zhang and Goodlett, 2004). Studies have also utilized proteomic techniques to identify oxidation-sensitive protein targets, a field referred to as redox proteomics (Butterfield et al., 2006; Ghezzi and Bonetto, 2003). Though identification of a full proteome of oxidant-sensitive mitochondrial proteins has previously proved challenging (Bailey et al., 2005), various thiol-specific labeling methods have been utilized to detect specific mitochondrial protein sulfhydryl groups susceptible to oxidative modification (Lin et al., 2002). Proteomic analysis of mitochondria has also turned attention to alterations in mitochondrial protein levels following oxidative stress both *in vitro* and *in vivo* (reviewed in Bailey et al., 2005).

In the presented studies, we used two primary methods of protein separation and detection, two-dimensional difference in-gel electrophoresis (2-D DIGE; **Chapter 3**) and 2-D gel electrophoresis combined with a radiolabeled ligand (**Chapter 4**). The methodologies we used

to detect covalent-modified proteins in **Chapter 4** are similar to previously described methods, particularly when comparing DIGE gels and their resulting autoradiograms in order to pinpoint modified proteins. Studies have used comparison of two separate 2-D gels, one immunoblotted for detecting oxidative modifications and a parallel protein stained gel used for spot picking and MS analysis. This method has been proven successful in identifying oxidized proteins in mouse models of neurodegeneration and human disease brain tissue (Castegna et al., 2002a; Castegna et al., 2002b; Castegna et al., 2003; Choi et al., 2004a; Poon et al., 2005b).

2-D DIGE is a relatively recent development in proteomic methodology, and solves many of the complications from doing only 2-D gel electrophoresis for quantitative study (Issaq and Veenstra, 2008). Specifically, DIGE allows for the direct comparison of two to three samples, depending on dye availability, within one gel. This eliminates the potential variability in protein migration encountered when comparing separate gels with standard 2-D gel electrophoresis, as well as allowing for higher reproducibility and confidence in quantitative analysis (Issaq and Veenstra, 2008; Unlu et al., 1997).

Our study (**Chapter 3**) was the first to examine the use of commercially available DIGE-compatible cysteine-reactive maleimide CyDyes in a minimal-labeling scheme and to compare those results to lysine-reactive NHS-ester CyDyes (Van Laar et al., 2008). Primarily intended for use in labeling scarce-abundance protein samples, Cys-CyDyes are typically used under saturation-labeling conditions (Shaw et al., 2003). However, when used under such conditions, others have noted that protein spot patterns did not match between Cys- and Lys-CyDye DIGE gels, complicating comparisons between the two methods (Chan et al., 2005; Shaw et al., 2003). Under minimal Cys-CyDye labeling conditions, our Cys- and Lys-CyDye DIGE gels were a near perfect match, as confirmed by MS identification of spots from both gels. It is possible that

saturation labeling of cysteines using CyDyes may have unintentional side effects such as cross-reactivity of the dyes with other amino acid residues, which may alter protein pI. In our experience, we found that the minimal-labeling technique for Cys-CyDyes may be a preferable method for DIGE detection.

One limitation to the methods we utilized was that observation of modifications, whether via DIGE comparison or covalent binding of ^{14}C -DA, was in part dependent on the basal abundance of the protein. This is particularly crucial for DIGE, where comparison of protein abundance in control and experimental samples is dependent on being able to visualize quantifiable amounts of the labeled protein. In the case of ^{14}C -DA covalently bound proteins, autoradiography allowed us to pinpoint the modified proteins, but we are mindful of the fact that the amount of observed radioactivity (based on the density of the spot on the autoradiogram) did not necessarily correlate to the amount of covalent modification. Intensity of a ^{14}C -DA-labeled spot may have been influenced by the overall abundance of the protein in question, and the ability to even see a modified protein was dependent on its abundance. For example, if half of the total amount of “Protein X” is modified, but there is very little Protein X in the sample, we may never detect a radiolabeled spot in the autoradiogram. However, if only 5% of “Protein Y” is modified, but “Protein Y” comprises 10% of the total protein in a sample, it may appear as a very strong target of modification. Resolving these issues can be a difficult and tedious task, and remains a major hurdle in some proteomics-based studies (reviewed in Ahmed and Rice, 2005). There are methods available, including fractionation of the proteome and, in the case of plasma samples, affinity chromatography-based depletion of high-abundance proteins, which may allow for concentration of scarce proteins (reviewed in Ahmed and Rice, 2005; Minden, 2007). There is also the option of using other proteomic methodologies such as liquid-chromatography-

MS/MS based shotgun proteomics, or MudPIT, with SILAC and ICAT differential labeling to examine complex protein mixtures (Jin et al., 2006; McDonald and Yates, 2002; Wu and MacCoss, 2002). However, these methods can suffer similar limitations based on protein abundance, and, since identification is based on sequencing of individual labeled peptides, are possibly confounded by incomplete labeling or the presence of multiple protein isoforms in samples (reviewed in Ahmed and Rice, 2005; McDonald and Yates, 2002; Wu and MacCoss, 2002).

Ultimately, the goal of proteomic studies is to give a picture of the multiple changes associated with a disease or toxin, and thus provide potential protein targets for further investigation. Sometimes the result can be overwhelming amounts of data. Validation, both from characterization of individual targets and reports of similar findings in repeated studies by others, is necessary to evaluate whether results from these studies are significant. To that end, I again point out that many of the proteins identified here as modified following exposure to DA oxidation are also reported with abundance alterations or oxidative modifications in other studies of neurological disease and disease models, including PD. Thus, our findings not only support previous results regarding a potential role for these proteins in oxidative stress and disease, but also encourage additional research to characterize these targets, such as mitofilin, with regard to DA toxicity and PD pathogenesis.

6.3 THE ROLE OF MITOFILIN IN TOXICITY AND DISEASE

When we first identified mitofilin as being modulated by DAQ-exposure in rat brain mitochondria, little was known about the protein beyond its structure and localization. Over the

course of our work, however, other studies began to associate alterations in mitofilin with neurological disorders (Myung et al., 2003; Omori et al., 2002), and found mitofilin was susceptible to oxidative and covalent modifications (Lai et al., 2008; Suh et al., 2004). Recently, mitofilin was reported to have a role in mitochondrial cristae maintenance (John et al., 2005) and associate with key mitochondrial protein import proteins (Xie et al., 2007). Overall, studies, including ours, now demonstrate that mitofilin is a crucial protein in normal mitochondrial function, a target of modification in times of cellular stress, and may have a key role in the pathogenesis of neurological diseases.

6.3.1 Mitofilin as a target for post-translational modification and altered expression

We identified mitofilin as a target for DA oxidation-induced decreases in abundance in isolated mitochondria and differentiated PC12 cells (see **Chapter 3**). Potential mechanisms for the reduction in mitofilin protein in isolated mitochondria are discussed in greater detail below. Decreased abundance of the protein in PC12 cells may result from similar mechanisms (discussed below) or decreased expression. In both cases, we found evidence via Western blot for DA-induced crosslinking and/or aggregation of the mitofilin protein. Additionally, we found evidence that DAQ covalently binds mitofilin in intact mitochondria *in vitro* (see **Chapter 4**), which may be contributing to the immunodetectable higher-molecular weight forms observed following DAQ exposure in **Chapter 3**. While we are the first to associate these modifications with exposure to DA or DAQ, other studies have established mitofilin as a target of oxidative and covalent modification following cellular stress. Oxidation of mitofilin cysteinyl residues has been reported following alcohol exposure in cells (Suh et al., 2004). Mitofilin was also found to be a target of poly-ADP-ribosylation in rat brain in a model of traumatic brain injury, suggesting

that modification of mitochondrial proteins is related to pathogenic over-activation of poly(ADP-ribose) polymerases, or PARP, and subsequent cell death (Lai et al., 2008). Though other proteins were also reported as modified in these proteomic analyses, the above studies show that mitochondrially-located mitofilin is susceptible to modification following cellular stress.

Models of cellular stress have also demonstrated effects on mitofilin expression. Downregulation of mitofilin was noted following photodynamic therapy in HL60 cells and MCF-7 cells (Kratassiouk et al., 2006; Magi et al., 2004), a cancer-treatment method that triggers oxidative stress and apoptosis. Only a few other proteins demonstrated altered expression in these studies, though multiple cellular proteins showed signs of oxidative modification through carbonylation (Kratassiouk et al., 2006; Magi et al., 2004). HL60 cells exposed to the apoptosis-inducing compound homoharringtonine (HTT) showed an initial decrease in mitofilin mRNA expression, followed by a rapid increase (6-fold) in mRNA expression within 6 hrs of treatment, one of only a few genes detected to behave in this manner (Jin et al., 2004). Such a response may suggest that the cells are attempting to recover following a toxic insult. Along this line, Navet et al. found that expression of mitofilin is significantly increased, along with altered expression of other mitochondrial proteins, in rat brown adipocyte cells during acclimation to colder temperatures, which requires high-energy usage (Navet et al., 2007). Increases in mitofilin may suggest an impact on mitochondrial structure or cristae shaping, which may affect mitochondrial function and energy production (Mannella et al., 2001).

Despite being a nuclear-expressed protein, mitofilin protein was also downregulated in myocytes with depleted mitochondrial DNA, a model of diabetic insulin resistance (Park et al., 2005; Park et al., 2006b). This model has demonstrated that a subset of nuclear expressed mitochondrial proteins is dysregulated following mitochondrial DNA depletion (Park et al.,

2006b). This is thought to be due to a mitochondrial stress-signaling pathway, affecting various factors including mitochondrial membrane biogenesis, intracellular signaling pathways, and nuclear transcription factor activity (Amuthan et al., 2002; Amuthan et al., 2001; Biswas et al., 1999; Biswas et al., 2003). Thus, alterations in mitochondrial DNA may be impacting mitochondrial-nuclear signaling and altering nuclear expression of mitochondrial proteins (Amuthan et al., 2002; Amuthan et al., 2001; Biswas et al., 1999; Biswas et al., 2003; Park et al., 2006b). As mitochondrial DNA deletions and mutations have been found to increase in neuronal mitochondria with age, particularly in aged and PD SN (reviewed in Biskup and Moore, 2006), it is possible that the mitochondrial-nuclear signaling system leads to decreased expression of vital proteins, such as mitofilin, impacting mitochondrial function and leading to neuronal dysfunction.

6.3.2 Altered mitofilin expression modulates cellular response to toxin exposure

After we identified mitofilin as a target of DA-induced modification, and knowing the proposed crucial role for mitofilin in mitochondrial cristae maintenance (John et al., 2005), we were encouraged to examine mitofilin in a more sophisticated model by altering mitofilin expression directly, through overexpression and RNAi techniques, in cultured dopaminergic cells. John et al. previously observed that overexpression had no observed effect on HeLa cell viability and mitochondrial function, while almost complete knockdown of mitofilin protein showed only slight increases in apoptosis with altered mitochondrial function (John et al., 2005). However, those experiments were not done in neuronal or dopaminergic cells. We found that modulation of mitofilin expression had little effect on viability in untreated cells, but altered the response of differentiated PC12 and SH-SY5Y cells to DA- and rotenone-induced toxicities. We

observed that increased mitofilin expression was protective and decreased mitofilin expression potentiated toxicity (see **Chapter 5**). This study provides the first evidence that altered mitofilin expression, specifically, can have an effect on cellular response to a stressor.

While our results give little clue as to the mechanism for the effects of modulating mitofilin expression on toxin-induced cell death, we can speculate as to why we see these effects based on what is known about mitofilin. As John et al. demonstrated, a knockdown of mitofilin led to severely disrupted mitochondrial cristae morphology, in association with mitochondrial functional deficits (John et al., 2005). Thus, it was anticipated that downregulating mitofilin would increase toxicity, which is what we observed with DA-induced toxicity in SH-SY5Y cells with reduced mitofilin expression. As we previously observed DA-induced decreases in mitofilin abundance in PC12 cells, we hypothesized that increasing expression of mitofilin prior to toxic insult would be protective against DA toxicity, which we observed in both PC12 and SH-SY5Y cells overexpressing mitofilin. Interestingly, we also observed protection against the mitochondrial toxin, rotenone. We have not yet evaluated whether rotenone alone can impact endogenous mitofilin levels. At present, we hypothesize that this protection is mediated through mitofilin-induced stabilization of mitochondrial cristae structure, potentially protecting against rotenone- and DA-toxicity induced disruptions in mitochondrial function by maintaining proper morphology and preventing loss of membrane potential. Protection may also be mediated through the potential role for mitofilin in mitochondrial protein import. Mitofilin was recently found to interact with a complex of proteins important for mitochondrial protein import (Xie et al., 2007). Thus, excess mitofilin may allow for continued mitochondrial protein maintenance and mitochondrial biogenesis related to the import of new proteins during cellular stress. Further studies, including examination of mitochondrial integrity using electron microscopy and

mitochondrial respiration in association with modulating mitofilin expression, will be necessary to evaluate these hypotheses.

It is important to note that the models used in our studies were acute toxicity. Thus, while they demonstrate mitofilin-associated effects against an abrupt insult, they do not provide insight to the effects of mitofilin increase or decrease over prolonged periods of time, or in concert with chronic exposure to stressors. Thus, a long-term neuronal culture model should be ideal to further evaluate the effects of altered mitofilin expression. Culture methods have been described for neuronal differentiation and long-term culture of SH-SY5Y (Constantinescu et al., 2007), which would accommodate the stable overexpressing SH-SY5Y cells we have already developed. Or, primary neuronal and organotypic cultures could be transfected to boost or knock down mitofilin expression. Not only would this provide *in vitro* insight to the effects of mitofilin modulation within neuronal cells over time, but also could be used in conjunction with already-established methods of chronic *in vitro* rotenone exposure (Sherer et al., 2003a; Testa et al., 2005).

The ultimate goal would be to move study of mitofilin into *in vivo* models to evaluate the role of mitofilin in dopaminergic survival and with respect to PD toxins. Viral-mediated delivery of constructs expressing mitofilin or mitofilin shRNA could be used not only to evaluate the effects of mitofilin expression *in vivo*, but also used in conjunction with established PD animal models, such as DA or rotenone toxicity. Transgenic models would also be valuable, particularly knockouts, if viable. If heterozygous knockouts with decreased expression could be developed, it would be interesting to examine whether they show early signs of aging or increased vulnerability to toxin exposure. For now, this is wishful thinking, but is a likely next step for investigating mitofilin with regard to neurodegenerative disease.

Despite enthusiasm for proposing additional studies, there is one important point to consider. At present, there is no reported link between mitofilin and PD, either familial or sporadic. This does not necessarily preclude a role for mitofilin, however. Thus, a logical next step in the study of mitofilin would be examination of PD patients, including CSF, platelets, or post mortem brain tissue, to look for alterations in mitofilin protein as compared to controls, whether that be abundance, distribution, or modification. If alterations were found, then experiments such as those described above would be well warranted in elucidating the role of mitofilin in PD. Even if minimal or no alterations were detected, the protection elicited by mitofilin in models of PD alone merit further study. Such work could eventually translate to new therapeutic avenues for PD involving mitofilin or its associated proteins.

6.3.3 A role for mitofilin in neuropsychiatric disorders, neurodegenerative disease, and PD

While there is currently no evidence that mitofilin plays a role in sporadic PD, alterations in mitofilin have been associated with other neurological disorders. Mitofilin expression, in association with other dysregulated proteins, was found to be decreased approximately 50% in post mortem Fetal Downs syndrome brain tissue as compared to control tissue (Myung et al., 2003), and decreased 72% in G93A-SOD1-expressing NSC34 cells, a cell line model of familial ALS, compared to wild type cells (Fukada et al., 2004). Alterations in protein sequence and structure of mitofilin have been tightly linked with altered neuronal function. Specifically, changes in mitofilin protein pI was noted in association with the senescence-accelerated mouse prone 8 (SAMP8) mouse model for age-related cognitive deficits and neuronal degeneration (Wang et al., 2008a), and in cortex from a seizure-sensitive strain of Mongolian gerbils (Omori

et al., 2002). Covalent DA modification of mitofilin may contribute to an altered structure and, thus, altered function in the mitochondria. These findings and our studies presented here demonstrate that mitofilin may play an important role in neurological disorders. While they suggest modification of mitofilin structure may be involved, they do not elucidate a clear mechanism for the effect of changes in mitofilin levels in cell function. It is important to note that no study prior to our work directly examined the manipulation of mitofilin in a neurodegenerative model or with regard to exogenous stressors. Our studies support a case for further examination into the function of mitofilin, and potential role of mitofilin in PD.

The effects of modulating mitofilin levels may be related to its interacting proteins. Mitofilin was found to associate with the schizophrenia risk factor protein disrupted in schizophrenia 1 (DISC1) (Millar et al., 2003), while a separate study found that truncated forms of DISC1 resulted in the formation of abnormal mitochondrial morphologies including enlarged rings and lariat-like structures (Millar et al., 2005). Recently, mitofilin was found to interact with a complex of proteins including SAM50 and metaxins 1 and 2, proteins associated with mitochondrial protein import mechanisms (Xie et al., 2007). The results of these studies suggest that mitofilin and its protein-protein interactions play an important role in normal mitochondrial function and morphology.

Perhaps the most remarkable characteristic that was noted in association with mitofilin loss is the severe reorganization of the mitochondrial cristae structure, resulting in concentric ring-like structures or whorls (John et al., 2005). Interestingly, similar structures have been noted in models of toxicity, disease, and cell death. Fornai and colleagues have noted the formation of cellular inclusions resembling membranous whorls following methamphetamine toxicity in PC12 cells, and in postsynaptic striatal neurons following 3,4-

methylenedioxymethamphetamine (MDMA) toxicity in mice (Ferrucci et al., 2002; Fornai et al., 2002; Lazzeri et al., 2006). The formation of whorls was thought to be associated with oxidative stress following excess DA release (Ferrucci et al., 2002; Fornai et al., 2002; Lazzeri et al., 2006). These whorl structures were immunopositive for ubiquitin, and were thought to represent an early sign of proteasome dysfunction and inclusion formation, though their origin is unknown (Ferrucci et al., 2002; Fornai et al., 2002; Lazzeri et al., 2006). Mitochondria exhibiting disrupted cristae structures resembling whorls, have also been observed in muscle tissue of mice expressing a mutant form of mitochondrial cytochrome c oxidase subunit I (Fan et al., 2008). Mitochondrial whorls, remarkably similar to those John et al. observed following mitofilin depletion, have also been observed with apoptosis in mature mouse oocytes in *in vitro* culture (John et al., 2005; Perez et al., 2007). The biological significance of these whorl structures is not known. However, their formation following knockdown of mitofilin, following toxic insult, and in association with apoptosis suggests they serve as a sign for mitochondrial dysfunction and cellular distress.

The association of mitofilin with DISC1 and other reported findings on altered mitofilin expression allow us to speculate on a potential role for this protein in mitochondrial dysfunction and neuropathology. The mitochondrial cristae structures are known to undergo reorganization in times of increased energy demands, cellular stress, and apoptosis (Mannella et al., 2001; Scorrano et al., 2002). In apoptosis, this may be partly due to peroxidation of inner membrane lipids, including cardiolipin, which facilitates the release of cytochrome c (Kagan et al., 2005; Kagan et al., 2006; Petrosillo et al., 2003; Petrosillo et al., 2001). It is possible that mitofilin is participating in this reorganization due to oxidation-induced alterations. Excess oxidants may modify mitofilin, altering its structure or targeting it for degradation, further allowing for cristae

destabilization and reorganization. As mitofilin also interacts with proteins connected to mitochondrial protein import (Xie et al., 2007), a loss of mitofilin could hamper efforts to recover from excessive protein damage and loss, setting up a deadly cycle of ROS generation, oxidative protein and lipid damage, ultimately leading to mitochondrial collapse. In dopaminergic neurons, this effect could be amplified by DA oxidation and modifications to mitofilin, contributing to the selective vulnerability of these neurons in toxin exposure or PD pathogenesis.

6.4 MITOCHONDRIAL AND CELLULAR PROTEINS AS TARGETS OF DA-INDUCED MODIFICATIONS

The studies comprising this thesis have identified several mitochondrial and cellular proteins as targets of DA oxidation-induced modifications. The potential impact of the DA-induced modifications associated with these proteins has been touched on in the **Discussion** sections of **Chapters 3** and **4**. In this section, I will expand upon these discussions, and attempt to link the seemingly varied pathways potentially affected by DA-induced oxidative modifications.

6.4.1 Changes in abundance vs. covalent modification of mitochondrial proteins following *in vitro* exposure to DAQ

Using proteomic methods, we observed two effects of exposure of isolated brain mitochondria to DAQ on mitochondrial proteins, protein loss and covalent modification (see

Chapters 3 and 4). We are not the first to observe that DA can covalently modify mitochondrial proteins *in vitro*. Exogenous DA applied to liver mitochondria was found to associate with multiple unidentified matrix proteins (Turan et al., 1989). Kahn et al. observed the formation of redox cycling quinone-proteins and aggregation of protein in fresh rat brain mitochondrial-synaptosomal fractions incubated with DA, which was prevented by reduced glutathione (GSH) (Khan et al., 2001). However, we are the first to identify specific mitochondrial protein targets of DA oxidation. These proteins, identified in **Chapters 3 and 4**, are targets for oxidative modification, and may be relevant to PD pathogenesis.

Availability and accessibility of reactive amino acid side chains is another factor to consider in the ability of DA to covalently modify proteins. It is important to note that while DAQ has extremely high affinity for and rapid reaction rate with reduced thiols (Tse et al., 1976), there is the possibility that it may bind other residues, such as lysine, depending on whether the side chain amine can act as a nucleophile under given conditions. Using a thiol-reactive compound that is accumulated by mitochondria, (4-iodobutyl)triphenylphosphonium, Lin et al. demonstrated that reactive thiols are accessible for covalent modification throughout the mitochondria, particularly thiol residues associated with the matrix faces of Complex I, II, and IV proteins (Lin et al., 2002). If DA or DAQ can be accumulated by mitochondria, they would have access to reactive thiols in multiple membrane and matrix proteins. Because thiols at physiological pH are the strongest nucleophiles in the cell, covalent modification by DA is likely occurring through, but not be limited to, thiol modification. Mass spectrometric sequencing analysis would be necessary to definitively answer this question by pinpointing the DAQ-modified residues, and is a logical next step in characterizing the DA-induced modifications to specific proteins identified in these studies.

One unresolved question from this work is whether the protein loss observed in our DIGE experiments is from protein aggregation or protein degradation. When we compared DIGE analysis to covalent modification following ^{14}C -DA exposure in isolated mitochondria, we found that while most proteins reduced in abundance following DAQ exposure are also covalently modified by DA, not all covalently modified proteins displayed a DAQ-induced reduction in protein amount (see **Chapter 4**). Mitochondrial proteins, including the matrix chaperone HSP60, the ubiquinol-cytochrome C reductase core protein 1 subunit of Complex III subunit, and aconitase all demonstrate covalent interaction with ^{14}C -DA, but show no loss of total protein following DAQ exposure based on DIGE analyses. This lack of complete overlap suggests that there may exist a selective process for removal of damaged proteins. Or it may attest to the affinity of some proteins to oxidatively crosslink and/or aggregate versus others. Both processes are a possibility within the intact mitochondria.

Mitochondria possess a proteolytic system of processing peptidases and proteases. These include processing peptidases involved in imported protein maturation and ATP-dependent proteases, including the Lon matrix protease, Clp-like matrix protease, and AAA-family inner membrane proteases (reviewed in Kaser and Langer, 2000). There is also the mitochondrial serine protease Omi/HtrA2. Whether this protein has proteolytic function within the mitochondria is uncertain. However, a mutation that inhibits its serine protease activity was recently linked to an increased risk of PD (Bogaerts et al., 2008; Strauss et al., 2005). Stable expression of human-related mutant forms in cells resulted in mitochondrial dysfunction (Strauss et al., 2005), suggesting a role for Omi/HtrA2 protease activity within mitochondria. Overall, the mitochondrial proteases and peptidases are not well characterized in mammals, and the functional significance of many mitochondrial proteases are not known (reviewed in Bulteau et

al., 2006; Kaser and Langer, 2000). Much of what is known about mitochondrial protease function comes from study of their homologues in yeast mitochondria. Such studies suggest that the ATP-dependent proteases are integral in mitochondrial protein quality control and serve a dual function, both in removal of misfolded and aberrant proteins, and in selective proteolysis related to mitochondrial biogenesis and morphology (reviewed in Escobar-Henriques and Langer, 2006; Kaser and Langer, 2000). Of these proteases, the Lon protease has received heightened attention for the discovery that mammalian Lon selectively proteolyzes the oxidized form of mitochondrial matrix aconitase (Bota and Davies, 2001; Bota and Davies, 2002). Knockdown of Lon protease expression in human lung fibroblasts resulted in disrupted mitochondrial morphology and increased apoptosis after 4 days (Bota et al., 2005). The surviving cells were characterized by severely depressed mitochondrial function and aberrant mitochondrial morphology, including the presence of intra-mitochondrial electron dense bodies as detected by electron microscopy, presumed to be accumulated oxidized protein (Bota et al., 2005). These findings suggest a crucial role for mitochondrial proteases in clearing damaged proteins.

Whether or not mitochondrial protein degradation is playing a role in protein loss in our *in vitro* study is uncertain. We did not observe any attenuation of protein loss by increasing the protease inhibitor cocktail (PIC) concentration during DA exposure, as reported in **Chapter 3**. But, as previously mentioned, treatment with PIC could be complicated by the dual membranes of the intact mitochondria, which may not allow entrance of the compounds in the cocktail into the mitochondria. Experiments with disrupted mitochondria would overcome this obstacle, but introduce new factors, such as release of non-membrane-bound proteins and the loss of our intact system, which is more physiologically relevant. Additionally, though the PIC contains

compounds for broad-spectrum protease inhibition, including cysteine-, serine-, and metalloproteases, there is no guarantee it is efficiently or specifically inhibiting all mitochondrial-based proteases. Further experiments titrating the level of protease inhibitors or using select types of inhibitors per experiment may shed light on the role of proteolytic degradation in our *in vitro* model.

We did observe evidence of SDS-insoluble protein interactions, however. Protein aggregation in DA-exposed mitochondria has previously been observed (Khan et al., 2001). However, that study merely observed extra, high molecular weight bands in SDS-PAGE gels of mitochondrial lysate, and did not delineate which proteins succumbed to aggregation (Khan et al., 2001). Using Western blot analyses to individually detect two altered proteins, mitochondrial creatine kinase (MtCK) and mitofilin, we detected multiple immunoreactive bands in the molecular weight range between the proteins of interest and the stacking gel only in samples exposed to DAQ (see **Chapter 3**). This would suggest DA oxidation-induced covalent crosslinking or other non-SDS-soluble interaction of proteins. In fact, the molecular weights of some of the bands detected (~85, 95, and 180 kDa for MtCK; ~180, and 250 kDa for mitofilin) are approximately multiples of the monomeric molecular weights of the proteins of interest (~42 kDa, MtCK; ~90 kDa, mitofilin), suggesting these may be homomeric crosslinked forms of the individual proteins. This is likely, considering MtCK is known to exist as an octameric protein (Speer et al., 2005; Wendt et al., 2003), and mitofilin has been suggested to form a homo-oligomeric complex (John et al., 2005).

More study is necessary to evaluate the predominant mechanism responsible for the loss of specific proteins following DA oxidation. It is also possible that degradation and aggregation are acting in parallel, an event that could have detrimental effects on mitochondrial viability *in*

vivo. Covalent DA modification and subsequent targeting of proteins for degradation automatically eliminates their function. If other mitochondrial functions, such as protein import, are impacted by DA oxidation, replacement of damaged proteins could be hindered. Rapid modification and targeting of proteins for degradation could also lead to aggregation as the mitochondrion's own proteolytic system becomes overwhelmed.

6.4.2 Energy production and energy management proteins as targets of DA-induced modifications

We observed DA-induced modifications to proteins involved in multiple steps of energy metabolism, including glycolysis (enolase and triosephosphate isomerase), the TCA cycle (isocitrate dehydrogenase, aconitase, succinyl-CoA ligase, and glutamate oxaloacetate transaminase 2), the ETC (30 kDa and 75 kDa Complex I subunits and Rieske Fe-S and ubiquinol-cytochrome C reductase core protein I complex III subunits) and ATP level maintenance (nucleoside diphosphate kinase A, creatine kinase (CK), and MtCK). Inhibition of TCA cycle proteins has previously been associated with PD (Mizuno et al., 1994), suggesting deficiencies in this system are related to disease pathogenesis. Similarly, oxidative modifications to CK and MtCK have been associated with disease and disease models (Boyd-Kimball et al., 2005; Butterfield et al., 2002; Castegna et al., 2002a; Choi et al., 2004a; Poon et al., 2004; Poon et al., 2005a), while alterations in mitochondrial ETC functions are believed to play a major role in PD pathogenesis.

CK enzymes convert cellular creatine into creatine phosphate by reducing ATP to ADP, thus storing the high-energy phosphate bond. This creatine phosphate store helps buffer against rapidly fluctuating energy usage, such as is found in muscle and neural cells (Eder et al., 2000).

The cytosolic form of CK occurs primarily as a dimer, (Muhlebach et al., 1994), while the mitochondrial form is primarily octameric, made up of dimer-dimer interactions (Gross and Wallimann, 1995; Schlegel et al., 1988; Schnyder et al., 1988). Oxidative modification of both CK and MtCK has previously been demonstrated (Butterfield et al., 2002; Castegna et al., 2002a; Dolder et al., 2001; Wendt et al., 2003). Previous studies have also shown that exposure of CK and MtCK to DA and DA quinones results in a decrease in enzymatic activity (Jiang et al., 2002; Maker et al., 1986; Miura et al., 1999).

Interestingly, the two Complex I subunits that we identified as targets of DA modification, the 30 kDa and 75 kDa subunits, have been reported to be targets of other biological processes that mediate cell death through disruption of mitochondrial function. Ricci et al. found that induction of apoptosis in HeLa cells led to a caspase-dependent cleavage and loss of the 75 kDa subunit, but not other subunits of Complex I (likely due to accessibility), mediating apoptosis-associated mitochondrial dysfunction (Ricci et al., 2004). Further, expression of a cleavage-resistant form of the 75 kDa subunit attenuated the apoptosis-associated loss of mitochondrial function, morphology, and ROS production (Ricci et al., 2004), suggesting a key role for this subunit in mitochondrial deficiencies associated with apoptosis.

Martinvalet et al. recently reported that the 30 kDa subunit of Complex I is a target for cleavage by the killer lymphocyte enzyme granzyme A (Martinvalet et al., 2008). Granzyme A initiates a caspase-independent apoptosis-like cell death in targeted cells that is dependent on mitochondrial disruption resulting in increased ROS and decreased mitochondrial membrane potential (Chowdhury and Lieberman, 2008). They demonstrated that the cellular effects of granzyme A may be mediated through disruption of complex I, and expression of a cleavage-resistant form of the 30 kDa subunit attenuated granzyme A-mediated cell death (Martinvalet et

al., 2008). However, the actual presence of granzyme A in the brain is uncertain. Overall, the effects elicited by directed cleavage of these two Complex I subunits suggest that their dysfunction or loss would contribute significantly to mitochondrial deficits and ROS production. The fact that the 30 kDa and 75 kDa subunits are targets of biological cell death pathways attests to their importance in cell survival. Though the nature of the DA modifications we identified on these two subunits is not yet certain, it is possible that DA oxidation of the 30 kDa and 75 kDa subunits could impact their function or interaction with other Complex I proteins, contributing to mitochondrial ETC dysfunction and cellular vulnerability.

Taken together, we can begin to see the potential impact of DA oxidation on the cellular energy system. If multiple proteins involved in ATP generation are targets for DA oxidation-induced damage or altered function, the result for dopaminergic neurons may be an adequate but limited energy system. Neurons could thus become stressed from a lifetime of slightly depressed mitochondrial respiration and increased ROS, creating an environment of increased oxidative stress and ongoing oxidative damage. These are characteristics often associated with aging in cells and mitochondria, particularly in the SN (Beal, 2002; Bulteau et al., 2006; Floor and Wetzel, 1998; Lenaz et al., 2002; Toescu et al., 2000). A limited energy system may also hinder the ability of the neurons to defend against or recover from acute stress. Such a state may cause dopaminergic neurons to be inherently more vulnerable to a pathogenic trigger, such as exposure to exogenous stressors like pesticides.

Modification of MtCK and nucleoside diphosphate kinase may also have a role in mitochondrial dysfunction beyond ATP level management. Both MtCK and the mitochondrial form of nucleoside diphosphate kinase (NDPK-D) localize to the intermembrane space, particularly contact sites, and interact with the lipids of the mitochondrial membranes, such as

cardiolipin (Dolder et al., 2001; Epand et al., 2007; Muller et al., 1985; Speer et al., 2005; Wegmann et al., 1991). Both proteins have also been shown to demonstrate lipid transfer properties, and may play a role in maintaining the mixed lipid compositions of the inner and outer mitochondrial membranes, key for maintaining mitochondrial structure (Epand et al., 2007). Evidence also suggests that the octameric structure of MtCK has a role in inducing and stabilizing contacts sites between the inner and outer membrane of mitochondria (Speer et al., 2005). Within contact sites, MtCK has been shown to interact with VDAC and adenine nucleotide translocase, proteins implicated in formation of the PTP (Beutner et al., 1998; Brdiczka et al., 1998; Dolder et al., 2001). Oxidative stress can result in dissociation of the octameric form of MtCK into dimers (Dolder et al., 2001; Speer et al., 2005; Vyssokikh and Brdiczka, 2003; Wendt et al., 2003). The dissociation is thought to facilitate opening of the PTP (Dolder et al., 2001; Vyssokikh and Brdiczka, 2003). Thus, oxidative modification of MtCK and nucleoside diphosphate kinase function and/or structure may result in mitochondrial membrane restructuring. As we have also demonstrated that mitofilin, a protein key in maintaining mitochondrial cristae structure, is susceptible to modification following DA oxidation, DA-induced damage to mitochondrial proteins may contribute to membrane reorganization, and ultimately promote mitochondrial dysfunction.

6.4.3 Mitochondrial and ER chaperones as targets of DA oxidation

The discovery of multiple cellular and mitochondrial protein chaperones as targets for covalent modification carries implications for the effects of DA oxidation on aberrant protein folding and aggregation in dopaminergic cells. While studies have demonstrated that DA can directly facilitate the aggregation of proteins, including alpha-synuclein (Conway et al., 2001)

and neurofilaments (Montine et al., 1995), modification and potential inactivation of chaperone proteins, such as ER-60, creates a situation in which proteins may aggregate due to misfolding. This could lead to induction of ER stress, which we observed in proteomic analysis of PC12 cells following DA toxicity (Dukes et al., 2008).

Of the chaperones found modified by DA oxidation, only mortalin/GRP75/mtHSP70 has been linked to PD. Mortalin/GRP75/mtHSP70 was identified to be significantly decreased in a proteomic analysis of PD brain SN mitochondria (Jin et al., 2006). A member of the hsp70 family of proteins and functioning primarily as a key chaperone in mitochondrial protein import and processing, mortalin/GRP75/mtHSP70 appears to be both a crucial and multifunctional protein in mitochondrial and cellular function, including management of oxidative stress and stability of mitochondrial morphology (reviewed in Wadhwa et al., 2002; Yaguchi et al., 2007). Functional inactivation of mortalin/GRP75/mtHSP70 in yeast results in aggregation of mitochondria by a mechanism independent of its protein import function (Kawai et al., 2001). Mortalin/GRP75/mtHSP70 also interacts with familial PD-associated DJ-1 protein, suggesting a role in regulation of oxidative stress (Jin et al., 2007). Recently, mortalin/GRP75/mtHSP70 was reported to interact with frataxin (Shan et al., 2007), suggesting a role in Fe-S cluster biogenesis and protein processing. Given the key roles mortalin/GRP75/mtHSP70 plays in the mitochondria, DA-induced modifications may result in dramatic effects on mitochondrial morphology and protein processing simply through impacting this protein. Additionally, we observed that DAQ exposure *in vitro* resulted in reduced abundance of mortalin/GRP75/mtHSP70. Perhaps DA oxidation plays a role in the loss of abundance of this protein associated with PD (Jin et al., 2006).

6.4.4 Cytoskeletal proteins as targets of covalent modification by DA

In both our mitochondrial and cellular models, we found cytoskeletal proteins to be vulnerable to covalent modification by DA. Actin and tubulin, were identified with covalent DA modification in our DAQ-exposed rat brain mitochondria, while actin and the actin-interacting protein tropomyosin were targets of covalent modification in DA-exposed SH-SY5Y cells (see **Chapter 3**). Previous studies have also demonstrated the susceptibility of cytoskeletal proteins to DA oxidation and oxidative stress relative to PD.

Montine et al. found that oxidized catechols could covalently crosslink neurofilaments (Montine et al., 1995). Aggregated neurofilaments have been associated with Lewy bodies, and both L & M neurofilament proteins have been found to be less abundant in PD brain SN (Basso et al., 2004). In agreement with our findings, previous work demonstrated that oxidized DA, induced by Fe^{2+} , bound to specific regions of skeletal muscle actin *in vitro* (Velez Pardo et al., 1995), further demonstrating the potential of actin as a target of dopamine oxidation. Though there are no other reports of DA-induced tubulin modifications, MPTP and rotenone toxicity, both of which generate excess ROS, have been shown to depolymerize tubulin microtubules in cell culture (Cappelletti et al., 1995; Feng, 2006). Additionally, exposure to quinones, including benzoquinone and juglone, resulted in decreased microtubule assembly and increased tubulin crosslinking *in vitro* and decreased neurite outgrowth in cultured hippocampal neurons (Santa-Maria et al., 2005b). Thus, exposure to and likely modification following oxidative stress can have drastic effects on the proper function of this critical cytoskeletal protein. Cytoskeletal modification may even have an impact on mitochondrial trafficking in the neuron, as mitochondria interact both directly with actin scaffolds for anchoring and with tubulin

microtubules for movement throughout the cell, respectively (Boldogh and Pon, 2006; Boldogh and Pon, 2007).

6.4.5 Antioxidant Enzymes and DA oxidation

Impaired antioxidant defense systems and oxidatively damaged antioxidant proteins have been previously associated with PD pathogenesis (Aoyama et al., 2000; Choi et al., 2006; Sian et al., 1994a; Sofic et al., 1992). Thus, it was no surprise that we found multiple cellular and mitochondrial antioxidant proteins to be targets of DA oxidation. Specifically, cytosolic peroxiredoxin II, SOD1 in SH-SY5Y cells, and mitochondrial peroxiredoxin III, and SOD2 in isolated mitochondria were covalent modification targets of DA, and SOD2 also showed altered abundance. We also found the PD-linked protein DJ-1 to be covalently modified in both cell culture and isolated mitochondria experiments. As discussed in **Chapter 4**, dysregulated expression and/or oxidative modification of each of these proteins has been associated with various diseases and disease models, including PD.

The significance of the identification of these proteins as targets of DA oxidation is emphasized by the knowledge that the human SN has a somewhat deficient antioxidant defense system in general. Glutathione levels in the brain were found to be at their lowest in the SN of normal human brain (Perry et al., 1982). These levels are even more decreased in PD brain (Pearce et al., 1997; Riederer et al., 1989; Sian et al., 1994a; Sofic et al., 1992). With this vital antioxidant system impaired, other systems may be heavily taxed. However, as we show here, those other mechanisms may be in danger of DA oxidation, and oxidized DA can bind glutathione, further jeopardizing the antioxidant defense of dopaminergic neurons. Indeed, study in SH-SY5Y cells and primary neuron culture found that induction of increased glutathione and

quinone oxidoreductase expression prevented toxicity from various toxins, including dopamine, 6-hydroxydopamine, 4-hydroxy-2-nonenal, and hydrogen peroxide (Jia et al., 2008). The presence of an adequate antioxidant defense system may be the key to decreasing DA oxidation and thus, preventing the effects of DA-induced toxicity.

6.4.6 Proteins linked to PD as targets of DA-induced modifications

Perhaps among the more significant findings of this thesis, with regard to PD, is the discovery of two PD-linked proteins, UCH-L1 and DJ-1, as targets for covalent modification by DA. Though other studies have found that these proteins are sensitive to oxidative stress and oxidative modification, direct DA modification has not previously been reported. DA modification has been reported for two other PD-linked proteins, alpha-synuclein (Conway et al., 2001) and parkin (LaVoie et al., 2005).

DA modification of alpha-synuclein was demonstrated to facilitate aberrant aggregation of the protein (Conway et al., 2001), while covalent modification of parkin, detectable in human brain tissue, inhibited protein function *in vitro* (LaVoie et al., 2005). As the results in this thesis are the first evidence of UCH-L1 and DJ-1 being covalently modified by DA, the effects of modification on function or structure of the proteins are largely unknown. However, recent studies in the Hastings laboratory demonstrate exposure of differentiated SH-SY5Y cells resulted in decreased UCH-L1 activity (Mishizen and Hastings, 2006). Potential impacts on function are also suggested by studies examining oxidative modification of the proteins. UCH-L1, a highly abundant protein in the brain (1-5% of soluble protein), is also a major target of oxidation and carbonyl formation in Alzheimer's disease and sporadic PD brains (Choi et al., 2004b), thus demonstrating a propensity of even the wildtype protein for modification in association with

neurodegeneration. Kabuta et al. demonstrated that carbonyl-modified UCH-L1 protein in COS-7 cells exhibited abnormal function and protein interactions similar to a mutant form of UCH-L1 associated with familial PD (Kabuta et al., 2008). Though the specific function is unknown, DJ-1 is considered to be an antioxidant protein by virtue of a reactive cysteine that becomes oxidatively modified in response to oxidative stress, and therefore has neuroprotective properties (Canet-Aviles et al., 2004). Though already present in mitochondria (Zhang et al., 2005), oxidation results in the translocation of cytosolic DJ-1 to the mitochondria (Canet-Aviles et al., 2004; Lev et al., 2008). The biological significance of this relocation, however, is unknown (Canet-Aviles et al., 2004; Lev et al., 2008). Like UCH-L1, increased oxidative modification of DJ-1 is also strongly associated with sporadic PD and Alzheimer's disease (Choi et al., 2006).

The fact that these four proteins, which are implicated in PD pathogenesis, are targets for activity or structural modification by DAQ suggests that dopaminergic neurons may be at greater risk in PD. Mutations in these proteins that are associated with PD often impact function and/or ability of the proteins to aggregate. Modification by DA may replicate these effects even in normal proteins, as is exemplified by the effect of carbonylation on normal UCH-L1 activity (Kabuta et al., 2008). Thus, DA oxidation may be conferring vulnerability to dopaminergic neurons by depressing or altering normal activity of these proteins.

6.4.7 The vicious cycle of DA oxidation in Oxidative stress, Mitochondrial Dysfunction, and Dopaminergic Vulnerability: A highly vulnerable population of proteins?

As stated above, and made apparent in other chapter discussions, many of the proteins we identified as modified appear to be common targets of oxidative modification and altered abundance in other models of oxidative stress and disease, and in diseases themselves. Many of

these proteins are cited *frequently* by other proteomic and oxidative stress studies examining protein modifications. Proteins including mitofilin, CK, MtCK, enolase, mortalin/GRP75/mtHSP70, VDAC, the 75 kDa and 30 kDa subunits of Complex I, the Rieske Fe-S and ubiquinol cytochrome c reductase core protein of Complex III, HSP60, glycerol 3 phosphate dehydrogenase, elongation factors, UCH-L1, tubulin, and actin are often found, either together or in various combinations, with altered protein expression or oxidative modification in proteomic studies associated with aging brain (Poon et al., 2005a; Poon et al., 2006; Weinreb et al., 2007), Alzheimer's disease (Abdi et al., 2006; Castegna et al., 2002a; Castegna et al., 2002b; Castegna et al., 2003; Sultana et al., 2006), ALS and ALS models (Fukada et al., 2004; Poon et al., 2005c), PD disease models (Jin et al., 2005; Palacino et al., 2004; Periquet et al., 2005; Poon et al., 2005b; Xun et al., 2007), and PD (Abdi et al., 2006; Basso et al., 2004; Jin et al., 2006). It is possible, as per an earlier discussion on the limitations of our own system, that the relative abundance of these proteins aids in their detection as targets of modification. However, the fact that they are cited in multiple diseases and models utilizing various detection methods would suggest that it is more complicated than sheer abundance.

The proteins we have identified in this work are potential targets of DA oxidation, and represent a particularly vulnerable subset of proteins that, in an environment of high oxidative stress, may be subject to modification, altered abundance, and may contribute to disease progression. Oxidative modification of these proteins may lead to inhibition of function, disrupted protein-protein interactions, or enhanced protein aggregation. Within the mitochondria, DA oxidation and damage of the protein targets discussed above would likely contribute to functional inhibition and structural instability. Ultimately, DA oxidation may initiate and/or contribute to a vicious cycle of oxidative stress, protein damage, and

mitochondrial dysfunction, weakening the cell and leaving it vulnerable to stressors. Thus, the potential of DA to contribute to the vulnerability of dopaminergic neurons in PD should not be ignored. Further investigation of the proteins identified as targets of DA oxidation in this thesis and their potential roles in PD pathogenesis may ultimately lead to a better understanding of disease progression and novel therapeutic avenues in the treatment of PD.

APPENDIX A

MITOCHONDRIA ASSOCIATED PROTEINS IDENTIFIED FROM DIGE GELS AND CORRESPONDING DECYDER ANALYSIS FOR CHAPTER 3

Table 5. Mitochondria associated proteins identified from DIGE gels and corresponding DeCyder analysis

Protein Spot	Protein	Protein Identification: (i) Top Hit for Protein Identification (ii) Highest Non-homologous Hit (Associated with Top Hit)	NCBI Database Accession #	Predicted Protein MW; pI	Protein Score; C.I.% **	Peptide Count	Percent Coverage	Peaks Matched/Peaks Searched	Cys-CyDye (% of control, \pm SEM)#	Lys-CyDye (% of control, \pm SEM)#
1	Gpd2	(I) Gpd2 protein [Rattus norvegicus] (ii) PREDICTED: similar to ARP2/3 complex 21 kDa subunit (p21-ARC) (Actin-related protein 2/3 complex subunit 3 [Rattus norvegicus])	gi 54035427 gi 27665454	80921.3; 6.18 19864; 8.8	101; 100% 26; 0%	17	31%	17/65	32.3 \pm 2.74*	46.6 \pm 4.55*
2	GRP75 (A)	(I) grp75 [Rattus sp.] (ii) Hox-D[Hox-4 [Rattus sp.]	gi 1000439 gi 30352212	73983.9; 5.87 7585; 10.48	243; 100% 31; 0%	27	53%	29/65	78.8 \pm 2.60*	73.7 \pm 3.30*
3	mitofilin	(I) similar to inner membrane protein, mitochondrial (mitofilin); motor protein [Rattus norvegicus] (ii) similar to Phosphoglycerate kinase, testis specific [Rattus norvegicus]	gi 34855983 gi 34859676	82304.9; 5.37 37007.1; 6.9	199; 100% 29; 0%	26	42%	27/65	35.2 \pm 2.39*	45.8 \pm 2.51*
4	NADH Deh 75kDa	(I) NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa [Rattus norvegicus] (ii) glutamyl aminopeptidase - rat (fragment)	gi 51858651 gi 2144073	79361.6; 5.65 15089.6; 7.03	245; 100% 36; 0%	30	49%	32/65	54.5 \pm 3.24*	54.1 \pm 3.48*
5	GRP75 (B)	(I) dnaK-type molecular chaperone grp75 precursor - rat (ii) hypothetical protein XP_237748 [Rattus norvegicus]	gi 2119726 gi 27666626	73698.8; 5.87 11111.7; 9.76	141; 100% 40; 0%	19	42%	20/65	73.3 \pm 3.41*	75.1 \pm 3.35*
6	HSP60	(I) unnamed protein product [Rattus norvegicus] (<i>HSP60 Protein</i>) (ii) similar to hypothetical protein E2300121.24 [Rattus norvegicus]	gi 1334284 gi 34862740	57889.7; 5.35 47293; 9.28	265; 100% 35; 0%	27	66%	32/65	101 \pm 2.08	93.5 \pm 0.771
7	Eno1	(I) Eno1 protein [Rattus norvegicus] (ii) similar to immunoglobulin heavy chain [Rattus norvegicus]	gi 50926833 gi 34935583	47098.2; 6.16 18662.4; 9.1	169; 100% 37; 0%	19	62%	22/65	127 \pm 7.63*	107 \pm 3.30
8	succinate-CoA ligase	(I) PREDICTED: similar to succinate-Coenzyme A ligase, ADP-forming, beta subunit [Rattus norvegicus] (ii) green fluorescent protein [Montastraea cavernosa]	gi 62661722 gi 32188168	50274.1; 7.57 25999.98; 7.05	156; 100% 44; 0%	20	52%	24/65	57.0 \pm 4.48*	57.9 \pm 2.22*
9	isocitrate deh. 3- α (A)	(I) isocitrate dehydrogenase 3 (NAD ⁺) alpha [Rattus norvegicus] (ii) similar to Lectin, galactose binding, soluble 1 [Rattus norvegicus]	gi 16758446 gi 34879568	39588; 6.47 16967.6; 9.78	89; 99.996% 34; 0%	13	37%	18/65	86.1 \pm 2.28	80.6 \pm 1.95*
10	isocitrate deh. 3- α (B)	(I) isocitrate dehydrogenase 3 (NAD ⁺) alpha [Rattus norvegicus] (ii) myosin heavy chain [Rattus norvegicus]	gi 16758446 gi 554476	39588; 6.47 19424.6; 5.29	133; 100% 25; 0%	17	46%	20/65	99.0 \pm 1.30	88.1 \pm 4.58
11	Tu translation elongation factor	(I) PREDICTED: similar to Tu translation elongation factor, mitochondrial [Rattus norvegicus] (ii) general amidase [Aspergillus fumigatus Af293]	gi 109462848 gi 70991685	49890.1; 7.23 62402.9; 6.13	209; 100% 63; 0%	21	65%	23/65	72.7 \pm 2.26*	59.1 \pm 1.43*
12	pyruvate carboxylase	(I) Pc protein [Rattus norvegicus] (ii) p21 protein [Rattus norvegicus]	gi 55716041 gi 56769	129694.7; 6.34 21229.5; 5.02	213; 100% 33; 0%	31	39%	32/65	63.9 \pm 5.90*	69.0 \pm 1.71*

Table 5 (continued)

Protein Spot	Protein	Protein Identification: (i) Top Hit for Protein Identification (ii) Highest Non-homologous Hit (Associated with Top Hit)	NCBI Database Accession #	Predicted Protein MW; pI	Protein Score; C.I.% **	Peptide Count	Percent Coverage	Peaks Matched/Peaks Searched	Cys-CyDye (% of control, ± SEM)#	Lys-CyDye (% of control, ± SEM)#
13	oxoglutarate dehydrogenase	(I) Similar to oxoglutarate dehydrogenase (lipoamide) [Rattus norvegicus]	gi 53734284	116221.4; 6.3	85; 99.99%	18	21%	18/65	50.5±4.91*	54.8±3.77*
		(ii) similar to Protein c20orf178 homolog [Rattus norvegicus]	gi 34859079	13220; 9.68	36; 0%					
14	RIKEN cDNA 2410002K23	(I) similar to RIKEN cDNA 2410002K23 [Rattus norvegicus]	gi 34868689	80410.8; 6.56	114; 100%	19	29%	20/65	74.0±7.00*	56.1±5.17*
		(ii) BM259 [Rattus norvegicus]	gi 15072458	16832.6; 4.99	30; 0%					
15	aconitase 2	(I) Aconitase 2, mitochondrial [Rattus norvegicus]	gi 38541404	85380; 7.87	307; 100%	34	51%	35/65	87.8±3.98	85.8±1.97
		(ii) acetylcholinesterase [Rattus norvegicus]	gi 25282401	68153.5; 5.9	43; 0%					
16	glutamate deh. 1	(I) glutamate dehydrogenase 1 [Rattus norvegicus]	gi 6980956	61377.3; 8.05	257; 100%	27	56%	34/65	101±5.29	96.5±1.37
		(ii) sema domain, immunoglobulin domain (Ig), short basic domain, sec [Rattus norvegicus]	gi 8394255	88751.9; 7.4	37; 0%					
17	MtCK (A)	(I) ubiquitous mitochondrial creatine kinase [Rattus rattus]	gi 57539	46999.3; 8.72	115; 100%	16	49%	18/65	18.1±2.71*	24.6±2.11*
		(ii) insulin receptor precursor [Rattus norvegicus]	gi 5230548	5078.5; 5.83	27; 0%					
18	MtCK (B)	(I) creatine kinase, mitochondrial 1, ubiquitous [Rattus norvegicus]	gi 60678254	46932.2; 8.58	128; 100%	17	54%	19/65	26.6±3.24*	41.4±2.93*
		(ii) hypothetical protein CdifQ_01003041 [Clostridium difficile QCD-32g58]	gi 85684359	22215.3; 5.7	42.5; 0%					
19	pyruvate deh E1 α	(I) pyruvate dehydrogenase E1 alpha form 1 subunit [Rattus rattus]	gi 57657	43168.6; 8.35	96; 99.999%	14	45%	18/65	90.1±5.72	95.4±2.71
		(ii) similar to L-lactate dehydrogenase A chain (LDH-A) (LDH muscle subunit) (LDH-M) [Rattus norvegicus]	gi 34851481	31563.8; 6.34	33; 0%					
20	aldehyde deh. 1, B1	(I) Aldehyde dehydrogenase 1 family, member B1 (predicted) [Rattus norvegicus]	gi 51858643	58101.6; 6.62	125; 100%	15	43%	17/65	N.D.***	64.2±3.94*
		(ii) glutamate dehydrogenase 1 [Rattus norvegicus]	gi 6980956	61719.4; 8.05	44; 28.477%					
21	VDAC2	(I) B-36 VDAC=36 kda voltage dependent anion channel [rats, hippocampus, Peptide, 295 aa]	gi 299036	31699.6; 7.44	56; 94.448%	8	40%	8/65	25.5±3.56*	31.7±3.25*
		(ii) ADAMTS protein [Rattus norvegicus]	gi 5929892	9102.7; 8.44	22; 0%					
22	fumarylacetoacetate hydrolase domain	(I) PREDICTED: similar to fumarylacetoacetate hydrolase domain containing 2A [Rattus norvegicus]	gi 34858672	40314; 8.49	122; 100%	15	56%	19/65	34.9±2.61*	43.9±2.09*
		(ii) Flot2 protein [Rattus norvegicus]	gi 53733398	38693.6; 5.26	32; 0%					
23	SOD2 (A)	(I) unnamed protein product [Rattus norvegicus] (<i>Superoxide dismutase 2</i>)	gi 56691	24667.6; 8.96	126; 100%	13	65%	13/65	65.2±3.06*	64.6±2.35*
		(ii) similar to ribosomal protein L30 [Rattus norvegicus]	gi 34855533	12708.8; 9.59	30; 0%					

Table 5 (continued)

Protein Spot	Protein	Protein Identification: (i) Top Hit for Protein Identification (ii) Highest Non-homologous Hit (Associated with Top Hit)	NCBI Database Accession #	Predicted Protein MW; pI	Protein Score; C.I.% **	Peptide Count	Percent Coverage	Peaks Matched/Peaks Searched	Cys-CyDye (% of control, ± SEM)#	Lys-CyDye (% of control, ± SEM)#
24	SOD2 (B)	(I) unnamed protein product [Rattus norvegicus] (<i>Superoxide dismutase 2</i>) (ii) similar to mesenchymal stem cell protein DSCD75 [Rattus norvegicus]	gi 56691 gi 27662044	24667.6; 8.96 23732.6; 9.14	59; 96.016% 23; 0%	8	41%	8/65	72.3±2.67*	N.D.****
25	F1-ATPase β	(I) Chain B, Rat Liver F1-ATPase [Rattus norvegicus] (ii) PREDICTED: similar to hypothetical protein MGC49942 [Rattus norvegicus]	gi 6729935 gi 27672993	51320.8; 4.95 17943.3; 6.74	289; 100% 29; 0%	26	86%	36/65	N.D.*****	122±5.41*

* Significance from control (100%), p<0.01, for proteins outside of the cutoff of 83.3-120% of control (1.2 fold change)

** Probability-based MOWSE score (Protein Score) and Protein Score Confidence Interval (C.I.) represent the top Protein Score and C.I. pairing obtained across all gels, Cys- and Lys-CyDye, in which the protein was confidently identified (n = 2-11)

*** Insufficient data for DeCyder analysis

**** Protein not identified via MS analysis in Lys-DIGE gels

***** Protein not identified via MS analysis in Cys-DIGE gels

Normalized fold change in fluorescence of DAQ sample compared to control as determined by DeCyder analysis, expressed as percent of control (100%) ± standard error of the mean (SEM)

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